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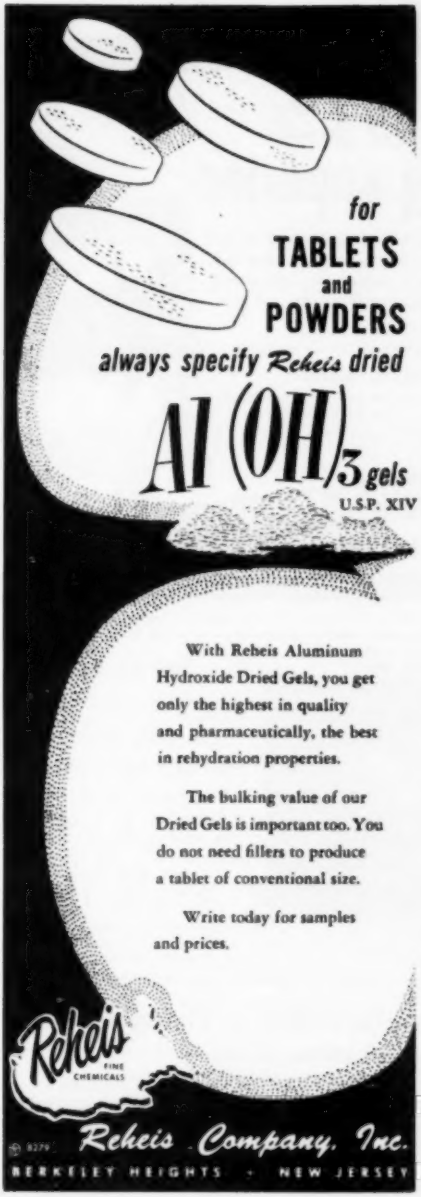
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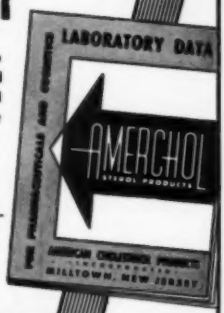

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLI — *AUGUST, 1952* — NUMBER 8 — CONSECUTIVE NO. 16

The Determination of Nydrazid*

By JOSEPH F. ALICINO†

Two simple and rapid methods for the assay of isonicotinic acid hydrazide are presented. One of these methods deals with the titration of the drug in anhydrous medium with perchloric acid, while the other has as its principle the oxidation by means of iodine in an alkaline solution.

THE FINDING (2) that Nydrazid (isonicotinic acid hydrazide) was effective in the treatment of tuberculosis prompted a search for methods which could be used for the assay of this compound.

Extensive studies in this laboratory on the range of applicability of methods using perchloric acid for titrations in a nonaqueous medium immediately suggested the use of this technique. Recent surveys (4, 7) and two recent articles (5, 6) which deal with specific application to certain pharmaceutical products indicated a wide acceptance of this type of assay. We had applied this procedure to the analysis of nicotinic acid, and its use was demonstrated in one of the articles cited (6). The hydrazide of isonicotinic acid was tested and the results indicated that both the pyridine nitrogen atom and the end amino group of the hydrazide were titratable. The potentiometric curve showed one sharp break, suggesting that both groups were of equal strength.

* Received March 1, 1952, from the Squibb Institute for Medical Research, New Brunswick, N. J.
† Thanks are due to Dr. Harry Yale for the preparation of specially purified Nydrazid used in this work.

EXPERIMENTAL

A sample of the compound (shown to be pure by elementary analysis) was dissolved in glacial acetic acid in a concentration of exactly 1.000 mg. ml. and titrated with 0.01 *N* perchloric acid; the results are shown in Table I. The ratio between weight of sample and volume of perchloric acid reveals that one milliliter of 0.01 *N* perchloric acid is equivalent to 0.6858 mg. of isonicotinic acid hydrazide.

For the assay of Nydrazid in aqueous solution, an alternate method was devised. In a recent text by Audrieth and Ogg (1), several methods for the determination of hydrazine were reported, based on the original work of Bray and Cuy (3). After a preliminary investigation of these methods, the so-called indirect iodine titration procedure was selected as the most reproducible in its application to the analysis of the hydrazide. The results indicated that in the oxidation of the compound, four equivalents of iodine are involved.

Table II shows the reproducibility of the method on a solution of the compound containing 100 mg. of Nydrazid per 100 ml. of water.

The two procedures have been worked out for relatively small amounts of material (2–5 mg.) but can be used for larger amounts by employing more concentrated titrating solutions and larger volumes of solvents.

Procedure 1.—A sample of the substance (3–5 mg.) is dissolved in about 5 ml. of glacial acetic acid. One drop of crystal violet indicator is added, and the violet-colored solution is titrated with standard 0.01 *N* perchloric acid which was prepared in glacial acetic acid. The end point is reached when a definite green coloration is produced. The end point may also be determined potentiometrically, a sharp

TABLE I

Weight of Sample, Mg.	Calcd. 0.01 *N* $HClO_4$	Found	% Found
3.00	4.38	4.35	99.4
		4.33	99.0
		4.38	100.0
		4.37	99.9
5.00	7.30	7.28	99.9
		7.33	100.6
		7.32	100.4
		7.25	99.4

TABLE II

Weight of Sample, Mg.	Calcd. 0.01 *N* Iodine, Ml.	Found	% Found
2.00	5.84	5.80	99.4
		5.85	100.3
		5.79	99.3
		5.82	99.8
4.00	11.68	11.72	100.4
		11.65	99.4
		11.66	99.9
		11.64	99.8

break occurring even with 0.01 *N* solution, especially when dioxane is added. The titrant may be standardized against a solution of potassium acid phthalate by the indicator method or potentiometrically (4, 6). A reagent blank is necessary but should not exceed 0.2 ml. of $HClO_4$. When using 0.1 *N* perchloric acid, the blank value is less than 0.05 ml. and can therefore be disregarded in the usual analysis.

Procedure 2.—A sample (2–4 mg.) is introduced into a 125-ml. glass-stoppered iodine flask and dissolved in 10–20 ml. of water, whereupon an excess of standard 0.01 *N* iodine solution is added. Five milliliters of 0.2 *N* NaOH are quickly added, and the flask is stoppered and allowed to stand for three to five minutes. Five milliliters of 0.5 *N* H_2SO_4 (or enough to make the solution approximately 0.1 *N* acid) is then added, and the excess iodine is titrated against 0.01 *N* thiosulfate solution, using starch as the indicator.

When dealing with large samples requiring the use of greater volumes and 0.1 *N* iodine, an amount of NaOH (preferably 2 *N*) sufficient to render the solution approximately 0.1 *N* with respect to the alkali concentration should be used. On acidification the concentration of acid should not be in excess of 1 *N*. Calculations are as follows:

$$\text{Ml. } I_2 \text{ Consumed} \times \text{factor} \times \frac{137.1}{4} \times 100 \Big/ \text{Sample weight} = \% \text{ Purity}$$

Table III shows typical values obtained by both of the proposed methods.

DISCUSSION

As is evidenced by the tables shown above, both methods of analysis give satisfactory results on pure substances. Since it may be presumed that Nydrazid will be combined for therapeutic purposes with other materials, care should be taken in the use of the proper method of analysis. For example, should a material such as magnesium stearate be incorporated in a preparation of the drug, adequate provision must be made for the presence of this substance by special treatment (6). In this case, the use of Procedure 2 would be indicated. Conversely, should an iodine-consuming constituent be present which is not basic in a nonaqueous medium, Procedure 1 would give more satisfactory results.

An idea of the accuracy of these methods and their utility for purity control may be gained from the following observation: the analysis of commercial Nydrazid samples generally indicated them to be 95% pure, no matter what method had been used in their preparation. On subsequent purification, this figure invariably rose to 99–100%, proving that the methods provide a true measure of the purity of this substance. Moreover, since 1 mg. of Nydrazid is equivalent to 1.46 ml. of 0.01 *N* perchloric acid and 2.92 ml. of 0.01 *N* iodine solution, it is evident that these methods are far superior, when used for purity control, to elementary analysis in regard to sensitivity.

TABLE III

Sample Weight, Mg.	0.01 *N* $HClO_4$, Ml.	0.01 *N* I_2, Ml.	Purity, % Method 1	Purity, % Method 2
4.457	6.47	....	99.5	...
3.350	4.88	...	99.9	...
3.612	5.25	....	99.6	...
4.043	5.88	....	99.7	...
3.373	....	9.85	....	100.1
2.614	....	7.60	....	99.6
2.137	....	6.22	....	99.8
4.882	....	14.23	....	99.9
190.1	27.65[a]	....	99.7	...
193.7	28.20[a]	....	99.8	...
113.5	....	33.15[b]	....	100.1
103.6	....	30.10[b]	....	99.8

[a] Ml. of 0.1 *N* ClO_4^-.
[b] Ml. of 0.1 *N* I_2.

REFERENCES

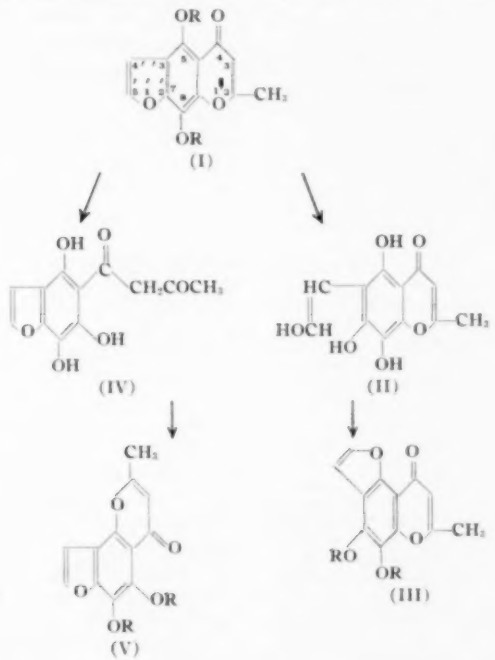
(1) Audrieth, L. F., and Ogg, B. A., "The Chemistry of Hydrazine," John Wiley & Sons, New York, 1951, p. 160.
(2) Bernstein, J., Lott, W. A., Steinberg, B. A., and Yale, H. L., *Am. Rev. Tuberc.*, **65**, 337(1952).
(3) Bray, C. W., and Cuy, E. J., *J. Am. Chem. Soc.*, **46**, 858(1924).
(4) Marcunas, P. C., and Riddick, J. A., *Anal. Chem.*, **23**, 337(1951).
(5) Pifer, C. W., and Wollish, E. G., THIS JOURNAL, **40**, 609(1951).
(6) Pifer, C. W., and Wollish, E. G., *Anal. Chem.*, **24**, 300 (1952).
(7) Riddick, J. A., *ibid.*, **24**, 41(1952).

Experiments with Khellin. II. The Synthesis of 5,6-Dimethoxy-2-methylfuro(2′,3′,7,8)chromone and Its Identity with Isokhellin*

By HAMED ABU-SHADY† and TAITO O. SOINE‡,§

Two syntheses for 5,6-dimethoxy-2-methylfuro(2′,3′,7,8)chromone are described, and this compound is identical with isokhellin. Preparative methods for isokhellinone and several new compounds incidental to the synthesis of isokhellin are also reported.

IN THE COURSE of attempts to prepare the parent dihydroxyfurochromone (I, R = H) from khellin (I, R = CH_3), Clarke and Robertson (1) found that, on being subjected to demethylation by means of boiling hydriodic acid, khellin gave rise to a somewhat resinous product from which only one crystalline phenolic substance could be isolated. Methylation of this substance yielded a dimethyl ether (m. p., 176°), isomeric, but not identical, with khellin, for which the name "isokhellin" was suggested.[1] The formation of isokhellin in this manner could take place during the demethylation process by either of the following routes, *viz.* (*a*) opening of the furan ring with the formation of II, followed by recyclization involving the C_5 hydroxyl group to give compound III (R = H), or (*b*) opening of the γ-pyrone ring to yield the intermediate diketone IV, and subsequent cyclization to form the alternative product V (R = H). As a result, isokhellin has been



* Received January 11, 1952, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis.

† Lecturer in Pharmacognosy, Fouad I University, Cairo, Egypt, and Fellow of the American Foundation for Pharmaceutical Education, 1949–1951.

‡ Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.

§ The authors wish to acknowledge with gratitude the financial assistance of the Egyptian Education Bureau and the American Foundation for Pharmaceutical Education.

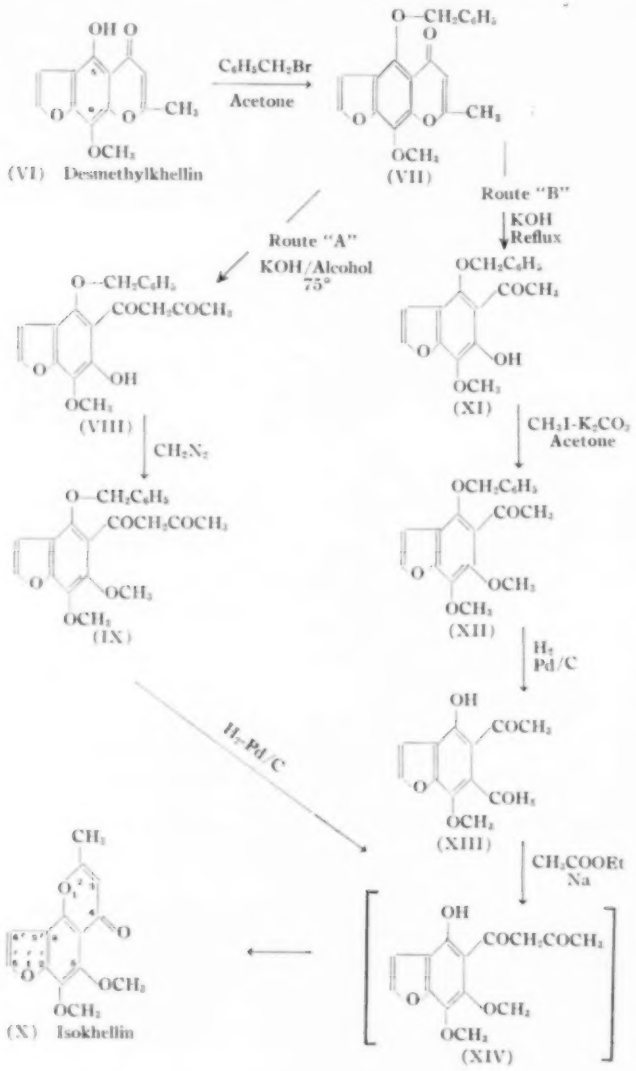
[1] The genetic nomenclature for khellin and isokhellin has been suggested by L. T. Capell, Associate Editor of *Chemical Abstracts* and co-author with A. M. Patterson of "Ring Index," in a letter received by one of us (T. O. S.) on February 19, 1952. Dr. Capell also suggested the following names for khellin and isokhellin, as named by the International Rules for Numbering Organic Ring Systems:

khellin—4,9-dimethoxy-7-methyl-5*H*-furo[3,2-*g*][1]benzopyran-5-one.

isokhellin—5,6-dimethoxy-2-methyl-4*H*-furo[2,3-*h*][1]benzopyran-4-one.

represented by either formula III (R = CH_3) or formula V (R = CH_3), of which, by analogy with the behavior of visnagin (2) under similar conditions, the former (III) was regarded by Clarke and Robertson as being the more probable. However, the latter change to V is not uncommon, as evidenced by the behavior of wogonin and its ethers on demethylation (3, 4).

In this paper the synthesis of 5,6-dimethoxy-2-methylfuro (2′, 3′, 7, 8)chromone (X) is described. This furochromone was found to be identical in every respect with isokhellin, as were the styryl derivatives of both X and isokhellin as prepared by the procedure of Clarke and Robertson. In an earlier communication (5), the authors of the present work have described the action of hydrobromic acid on khellin. When refluxed with about 40 per cent hydrobromic acid (sp. gr. 1.38), demethylation of one of the methoxyl groups was effected to yield the phenolic substance "desmethylkhellin" (VI). Although the evidences presented suggested strongly that this demethyla-



tion process involved the C_5 methoxyl group rather than the C_8 methoxyl group, unequivocal proof was lacking.

To prove the position of the phenolic hydroxyl as being at C_5 rather than at C_8, the following evidence is provided. Desmethylkhellin was refluxed with benzyl bromide and potassium carbonate in acetone to convert it smoothly and almost quantitatively to the colorless benzyl ether (VII). Treatment of benzyldesmethylkhellin with an alcoholic solution of potassium hydroxide under mild conditions resulted in the opening of the γ-pyrone ring to yield the corresponding diketone (VIII). This diketone gave a red coloration with ferric chloride solution. Treatment of this diketone with diazomethane effected the methylation of the phenolic hydroxyl group, but did not seem to affect the enolic hydroxyl group that presumably was present, as indicated by the positive ferric reaction given by the methylated compound. However, neither the diketone nor its methyl ether (IX) formed a copper coordination compound, as might be expected on treatment of an alcoholic solution with an aqueous solution of copper acetate. Treatment of the diketone methyl ether with 2,4-dinitrophenylhydrazine yielded a crystalline mono-derivative. Hydrogenolysis of the diketone methyl ether in alcohol in the presence of 10 per cent palladium-on-charcoal unexpectedly gave rise to colorless 5,6-dimethoxy-2-methylfuro-(2′,3′,7,8)chromone (X), m. p. 180°. This product was found to be identical with isokhellin prepared according to the directions of Clarke and Robertson. Condensation of the above furochromone with piperonal in the presence of sodium methoxide gave rise to a yellow styryl derivative, m. p. 226°, identical with the styryl derivative of isokhellin. On standing in the refrigerator for one week a concentrated alcoholic solution of the diketone methyl ether (IX) deposited crystals which were also found to be identical with isokhellin (m. p. and mixed m. p.). If the phenolic hydroxyl group of desmethylkhellin had been at the C_8 position, it would have been impossible to recyclize the benzyl ether following hydrolysis, methylation, and hydrogenolysis because both phenolic hydroxyls adjacent to the acetoacetyl side chain, either of which must be involved in recyclization, would appear in the intermediate compound XIV as methoxyl groups. The ultimate recyclization of the diketone methyl ether under the above conditions eliminates any doubt concerning the structure of desmethylkhellin as it was first suggested by the authors, i. e., 5-hydroxy-8-methoxy-2-methylfuro(3′,2′,6,7)chromone.

Alternatively, isokhellin was synthesized via another route. The benzyl ether of desmethylkhellin was hydrolyzed by potassium hydroxide in dilute alcoholic solution to the corresponding khellinone-type structure (XI). The latter was methylated by the methyl iodide-potassium carbonate method. Hydrogenolysis of the resulting methyl ether (XII) gave rise to isokhellinone (XIII). The reaction of isokhellinone with ethyl acetate in the presence of metallic sodium resulted in the formation of ω-acetylisokhellinone (XIV). The latter compound, although not isolated, readily underwent conversion to isokhellin on standing in an alcoholic solution.

EXPERIMENTAL

Method A

5-Hydroxy-8-methoxy-2-methylfuro(3′,2′,6,7)-chromone (Desmethylkhellin, VI).—This phenol (m. p. 201°) was prepared from khellin by the action of hydrobromic acid (sp. gr. 1.38) in almost quantitative yield by the method of Abu-Shady and Soine (5).

5-Benzyloxy-8-methoxy-2-methylfuro(3′,2′,6,7)-chromone (Benzyldesmethylkhellin, VII).—Desmethylkhellin (3 Gm.) was refluxed for twenty-four hours with benzyl bromide (3 cc.), anhydrous potassium carbonate (10 Gm.), and acetone (100 cc.). The reaction mixture was filtered from the potassium salts and washed four times with small volumes of acetone. The combined filtrate and washings were evaporated to a small volume under a hood and then allowed to crystallize in the refrigerator. The crude benzyl ether (3.5 Gm.) was collected, and after three recrystallizations from alcohol melted at 146°.

Anal.—Calcd. for $C_{20}H_{16}O_5$: C, 71.4; H, 4.8. Found: C, 71.8; H, 4.8.

5-Acetoacetyl-4-benzyloxy-6-hydroxy-7-methoxy-coumarone (VIII).—A solution of potassium hydroxide (6 Gm.) in 100 cc. of alcohol at 75° was poured on 2 Gm. of benzyldesmethylkhellin. The mixture was shaken at intervals over a period of one-half hour, or until the benzyl ether completely dissolved without further heating. The resulting dark-red solution remained clear when 2 cc. was diluted with a large volume of water. The alkaline solution was then rendered acidic by the addition of acetic acid, diluted with 400 cc. of water, and kept in the refrigerator overnight. The yellow crystals (1.85 Gm.) obtained were recrystallized from a mixture of benzene and petroleum ether (b. p. 30–60°) to give a constant melting point of 128°. This compound gave a red ferric reaction.

Anal.—Calcd. for $C_{20}H_{16}O_6$: C, 67.8; H, 5.1. Found: C, 67.4; H, 5.1.

5-Acetoacetyl-4-benzyloxy-6,7-dimethoxycoumarone (IX).—An excess of an ethereal solution of diazomethane (derived from 3 Gm. of N-nitrosomethylurea) was added to a solution of 5-acetoacetyl-4-benzyloxy-6-hydroxy-7-methoxycoumarone (5 Gm.) in a mixture of 2 cc. of methyl alcohol and 30 cc. of ether. The reaction was immediately apparent, as shown by the evolution of nitrogen. After standing overnight, the ether was distilled from the

solution. The residue, a reddish-colored oil, was not amenable to crystallization. An alcoholic solution of the oil gives a green ferric reaction which changes to red on standing or by gentle warming. A portion (0.25 Gm.) of this oil in alcohol (15 cc.) was added to a solution of 0.5 Gm. of 2,4-dinitrophenylhydrazine in a mixture of 2 cc. of hydrochloric acid and 15 cc. of alcohol. The hydrazone, which first separated as a dark brown oil, was crystallized from a large volume of ethyl acetate, and was obtained as brick-red needles, m. p. 235–236°.

Anal.—Calcd. for $C_{27}H_{24}O_9N_4$: C, 59.1; H, 4.4. Found: C, 59.3; H, 4.0.

5,6-Dimethoxy-2-methylfuro(2′,3′,7,8)chromone (Isokhellin, X).—A solution of 5-acetoacetyl-4-benzyloxy-6,7-dimethoxycoumarone (IX) (2 Gm.) in 100 cc. of alcohol, together with 0.25 Gm. of a 10% palladium-on-charcoal catalyst was reduced in a Parr low-pressure hydrogenation apparatus under an initial pressure of 40 lb./sq. in. of hydrogen at room temperature. The reaction was complete in about one-half hour. The catalyst was removed by filtration and the filtrate was distilled under reduced pressure to a volume of 10 cc. and then kept in the refrigerator until it crystallized. One-half gram of crystals was obtained which, after repeated crystallization from ether, melted at 180°. The crystals were colorless prismatic needles and gave no ferric reaction. A mixture of these crystals with isokhellin [prepared according to the procedure of Clarke and Robertson (1)] melted at 180°. When heated with sulfuric acid, these crystals displayed the same color change as described by Clarke and Robertson for isokhellin. When warmed with a concentrated potassium hydroxide solution, the crystals gave a red coloration. A similar color reaction was observed when isokhellin was treated in the same way.

Anal.—Calcd. for $C_{14}H_{12}O_5$: C, 64.6; H, 4.6. Found: C, 64.6; H, 4.9.

A mixture of the above crystals (0.3 Gm.), piperonal (0.3 Gm.), sodium methoxide (from 0.2 Gm. of sodium), and methanol (20 cc.) was refluxed for fifteen minutes and then kept at room temperature for twenty-four hours. Crystallization of the resulting yellow product from dilute acetic acid gave the styryl derivative in slender yellow needles (0.25 Gm.), m. p. 226°. A mixed melting point with the corresponding styryl derivative of isokhellin did not depress the melting point.

Anal.—Calcd. for $C_{22}H_{16}O_7$: C, 67.3; H, 4.1. Found: C, 67.1; H, 4.1.

When a solution of 5-acetoacetyl-4-benzyloxy-6,7-dimethoxycoumarone (IX) (5 Gm.) in 10 cc. of alcohol was allowed to stand for a week in the refrigerator, yellowish crystals (2 Gm.) were deposited which, after two crystallizations from alcohol, melted at 180° and which, by mixed melting point, were found to be identical with isokhellin.

Method B

5-Acetyl-4-benzyloxy-6-hydroxy-7-methoxycoumarone (XI).—Benzyldesmethylkhellin (5 Gm.) was refluxed with a solution of potassium hydroxide (5 Gm.) in a mixture of 35 cc. of alcohol and 15 cc. of water for one hour. The dark red solution was carefully acidified with dilute hydrochloric acid, diluted with a large volume of water, and left in the refrigerator to crystallize. The red semicrystalline mass obtained (4.5 Gm.) was readily crystallized from methyl alcohol to give yellow prisms, m. p. 102° (depressed by khellinone to 70°). The alcoholic solution of these crystals gave a dark-green ferric reaction.

Anal.—Calcd. for $C_{18}H_{16}O_5$: C, 69.2; H, 5.2. Found: C, 68.9; H, 5.2.

5-Acetyl-4-benzyloxy-6,7-dimethoxycoumarone (XII).—Two grams of 5-acetyl-4-benzyloxy-6-hydroxy-7-methoxycoumarone (XI) was refluxed with methyl iodide (4 cc.), potassium carbonate (10 Gm.), and acetone (50 cc.) for forty-eight hours, or until the solution ceased to give a green ferric reaction. The solution was then filtered and the acetone removed by distillation. The oily residue was then distilled at 170–180°/1 mm. or, better still, dissolved in ether and shaken with successive portions of 2% potassium hydroxide solution to remove any unconverted phenol. The ethereal solution was then washed with water, dried with anhydrous sodium sulfate, and distilled. 5-Acetyl-4-benzyloxy-6,7-dimethoxycoumarone (2 Gm.) was thus obtained as a colorless oil. One-half gram of this oil was refluxed for thirty minutes with hydroxylamine hydrochloride (0.5 Gm.), dry pyridine (0.5 cc.), and alcohol (15 cc.). The alcohol was then distilled from the solution and the residue was treated with an excess of ice-cold water. The oxime separated as an oil, which crystallized after standing for one week. When recrystallized from dilute alcohol, the oxime melted at 119–120°.

Anal.—Calcd. for $C_{19}H_{19}O_6N$. C, 66.9; H, 5.6. Found: C, 67.2; H, 5.6.

5-Acetyl-6,7-dimethoxy-4-hydroxycoumarone (Isokhellinone, XIII).—One gram of palladium-on-charcoal (10%) was added to a solution of 5-acetyl-4-benzyloxy-6,7-dimethoxycoumarone (XII) (5 Gm.) in alcohol (100 cc.), and the mixture was reduced in a Parr low-pressure hydrogenation apparatus. The reaction was completed in less than an hour. The yellow solution was filtered from the catalyst and the alcohol removed by distillation. The residual yellow oil crystallized on standing overnight. When recrystallized twice from methyl alcohol, isokhellinone (3.2 Gm.) had a melting point of 56°, and gave a dark-green ferric reaction.

Anal.—Calcd. for $C_{12}H_{12}O_5$: C, 61.0; H, 5.1. Found: C, 60.8; H, 5.2.

The 2,4-dinitrophenylhydrazone formed prismatic brick-red needles, m. p. 168° from alcohol.

Anal.—Calcd. for $C_{18}H_{16}O_8N_4$: C, 51.9; H, 4.3. Found: C, 52.2; H, 3.8.

5,6-Dimethoxy-2-methylfuro(2′,3′,7,8)chromone (Isokhellin, X).—To a solution of isokhellinone (5 Gm.) in 20 cc. of ethyl acetate was added 3 Gm. of sodium in small pieces. When the reaction had subsided, the mixture was refluxed on a steam bath for fifteen minutes and then kept at room temperature overnight. Ether was then added to the dark-brown, almost solid mixture, and the suspension poured on crushed ice. Water was then added to bring the volume of the solution to 500 cc. The solution was extracted with ether several times, acidified with acetic acid, and then extracted repeatedly with small portions of ether. The ether extractions of the acidified solution were combined, washed with water, dried, and then evaporated. The dark-brown oily residue was dissolved in a small amount of alcohol and left to crystallize at room temperature. The solid (2.5 Gm.) was col-

lected, and after two crystallizations from alcohol was obtained as colorless prismatic needles, m.p. 180° with a negative ferric reaction. It gave a red color with potassium hydroxide pellets and a little water, and a mixed-melting-point determination with isokhellin showed no melting-point depression.

SUMMARY

1. 5,6 - Dimethoxy - 2 - methylfuro(2′,3′,7,8)-chromone has been synthesized by two routes, and has been shown to be identical with the isokhellin of Clarke and Robertson.

2. It has been shown that khellin, when refluxed with hydriodic acid, undergoes rearrangement of the γ-pyrone ring, rather than of the furan ring, as is the case with visnagin.

3. The C_5 (as against C_8) location of the phenolic hydroxyl group in desmethylkhellin has been proved.

4. Isokhellinone has been prepared and described.

5. A number of new compounds incidental to the synthesis of isokhellin have also been described.

REFERENCES

(1) Clarke, J. R., and Robertson, A., *J. Chem. Soc.*, **1940**, p. 302.
(2) Clarke, J. R., Glaser, G., and Robertson, A., *ibid.*, **1948**, p. 2260.
(3) Shah, R. C., Mehta, C. R., and Wheeler, T. S., *ibid.*, **1938**, p. 1555.
(4) Hattori, S., *Ber.*, **72B**, 1914(1939).
(5) Abu-Shady, H., and Soine, T. O., THIS JOURNAL, **41**, 325(1952).

The Effects of Cobalt, Acetates, Ascorbic Acid, and Cholesterol on Growth and Glycoside Biosynthesis in *Digitalis purpurea**,†

By DANIEL P. N. TSAO‡ and H. W. YOUNGKEN, Jr.§

Various concentrations of cobalt chloride, ascorbic acid, potassium and sodium acetates, and cholesterol were fed to digitalis plants growing in the field and in hydroponic culture solution under controlled greenhouse conditions. Certain inhibitory growth responses are described following the use of the above-mentioned compounds. Cobalt chloride in the concentration of 10 p. p. m. produced marked inhibition of total glycoside production. Potassium and sodium acetates in concentrations of 0.4 and 0.2 per cent did not produce any significant effects on the biosynthesis of total glycosides. Ascorbic acid in the concentrations of 20 and 40 p. p. m. and cholesterol in concentrations of 0.5 and 2 per cent stimulated glycoside biosynthesis.

REPORTS in the literature have indicated that certain chemical substances, such as cobalt salts, various acetates, ascorbic acid, and steroids influence carbohydrate formation in plants. Ahmad, *et al.* (1), showed that cobalt chloride in the concentration of 0.005 per cent (50 p. p. m.) produced a retardation of chlorophyll and ascorbic acid synthesis in Bengal gram. Schwarrer and Schropp (2) found that a concentration of 300 p. p. m. cobalt sulfate produced a significant decrease in growth of cereal plants. Krotkov and Barker (3) showed that acetate ions were utilized for carbohydrate synthesis in tobacco leaves. Mapson and Cruickshank (4) further indicated that potassium acetate in a concentration of 0.02 *M* increased the synthesis of ascorbic acid in cress seedlings by 56 per cent. Sugawara (5) reported that in a study of the formation of ascorbic acid in spinach, barley, wheat, and other plants, the greatest accumulation of ascorbic acid occurred at the same time as the highest concentration of carbohydrates in the leaves. Other workers (6–11) have also shown that there is a relationship between ascorbic acid concentration and carbohydrate synthesis in plant growth.

During recent years there have been various reports on plant growth effects produced by steroid compounds. In one instance where digitalis was used, Much (12) increased the

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† This paper is based on Part II of a thesis presented to the Graduate School, University of Washington, by Daniel P. N. Tsao in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
‡ Fellow of American Foundation for Pharmaceutical Education at the University of Washington.
§ Associate Professor and Chairman for Pharmacognosy, College of Pharmacy, University of Washington, Seattle 5, Wash.

growth and metabolism by feeding plants with a 2 per cent cholesterol mixture. However, corresponding effects on glycoside biosynthesis were not studied. Brady and Gurin (13) have shown that a "2-carbon fragment" might be transformed into acetic acid and vice versa, and that such a fragment could be used for the biosynthesis of the steroid.

In so far as digitalis glycosides are thought by some to be associated with carbohydrate metabolism, it would appear that a stimulation or inhibition of growth, and of carbohydrate and steroid synthesis such as by the compounds mentioned above might influence the formation of these glycosides *in vivo*. Regulatory compounds which might affect digitalis glycoside production would be of some value during efforts to interpret pathways for its biosynthesis. This study deals with the effects of high titers of cobalt, acetates, ascorbic acid, and cholesterol on the growth and glycoside biosynthesis in this plant.

MATERIALS AND METHODS

Digitalis purpurea plants of approximately the same size and age were grown in the field and in culture solution bottles according to the method previously described by Tsao and Youngken (14). Chemically pure cobalt chloride, potassium acetate, ascorbic acid, and cholesterol were fed to plants growing in field plots and in a Hoagland culture solution (14), by a variety of methods herein described. One and one-half liter wide-mouth bottles painted black were used for solution cultures.

Representative leaves were selected during various periods of collection for the study of growth measurements and leaf size, and rates of growth were compared with those of controls.

Tinctures were made from dried and powdered leaves according to the U. S. P. XIV procedure (15). These were analyzed for total glycosides by a modified Knudson-Dresbach colorimetric method (14) and spot comparisons were made with a modified U. S. P. XIV biological procedure (15), during which pigeons weighing from 400 to 600 Gm. were used. All material was compared in potency with that of a U. S. P. Reference Digitalis Powder.[1] Degrees of significance were calculated by the conventional student "*t*" test (16) for analyses of this kind.

EXPERIMENTAL

Effects Induced During Field Growth Conditions.—Seedlings of *Digitalis purpurea* grown from pure strain seed material were transplanted into experimental plots in the field of the Drug Plant Gardens, University of Washington. Plants were arranged into convenient rows and spaced at least two feet apart. Plants of approximately the same size and leaf development were used. These were placed into the field in March, 1948, and divided into seven groups. Feedings with chemical substances were commenced 69 days later during the first week of June, 1948. By this time each plant had developed adequate growth and was considered to be of suitable size for further testing. Cobalt chloride (50 p. p. m.), potassium acetate (0.02 *M* or 0.2%), and ascorbic acid (10 p. p. m.) were applied by pouring approximately 100 cc. of aqueous solutions of these compounds onto the leaf and crown portions of the plant. Care was taken to saturate all parts of each leaf. Plants were treated once every two weeks during clear weather for four treatments. Untreated or control plants were watered with the same quantity of tap water in the same manner. Because of the insolubility of cholesterol in water, a 10% ointment preparation was employed. The ointment base consisted of equal parts of petrolatum U. S. P. and liquid petrolatum U. S. P. This preparation was applied to one surface of the petiole portions of each plant with a camel's-hair brush. Applications were made two weeks apart for two treatments. Control plants for the latter group were similarly painted with a mixture of equal parts of U. S. P. petrolatum and liquid petrolatum.

Growth Results.—During the growing period it was noted that plants treated with cobalt chloride generally were slower in development than controls and other treated groups. Some mottling of leaves occurred. No significant differences were noted among potassium acetate and ascorbic acid-treated plants. Significant growth effects were noted among plants treated with cholesterol. Shortly following the first application, leaves showed marked thickening and darker green coloration and curling. Ointment control plants generally exhibited more toxic effects following 41 days of treatment than did those treated with cholesterol. At the end of 64 days of treatment it was observed that approximately 16% of cholesterol-treated plants and 6% of ointment control plants had survived.

From these findings it was concluded that digitalis plants growing in the field would tolerate cobalt chloride, potassium acetate, and ascorbic acid in the quantities applied. Cobalt chloride, however, showed indication of producing slight toxic responses. Ointment applications of cholesterol and its ointment control, on the other hand, were significantly toxic. In the latter case, 10% cholesterol ointment was somewhat less severe in toxicity than the petrolatum ointment used as a control.

Glycoside Analyses.—Representative leaf material was collected periodically from each experimental field plot. These were dried and tinctures were then prepared for glycoside study. Periodic leaf collections were made from each of the treated and untreated groups at the end of 18, 41, 64, 71, and 83 days of treatment. Results are shown in Table I.

Noticeable differences in total glycoside yields were observed following colorimetric analysis of plants treated with cobalt chloride, cholesterol ointment, and ointment controls. Cobalt and petrolatum appeared to inhibit glycoside formation. Cobalt treated plants gave glycoside yields which were averagely lower than those of controls and other treated plants. For example, leaf material from cobalt treatments assayed averagely 20.50%

[1] The authors wish to acknowledge the generous assistance given during the biological testing program by the Department of Pharmacology, School of Medicine, University of Washington.

TABLE I.—RESULTS OF GLYCOSIDE ANALYSIS FOLLOWING CHEMICAL FEEDINGS OF DIGITALIS PURPUREA DURING FIELD GROWTH

Experimental Groups	No. of Plants	Days of Treatment — Activity (Per Cent Potency in Terms of U. S. P. Digitalis Reference Standard)[a] 18	41	64	71	83	Days of Treatment — Percentage Difference (in Comparison with Controls) 18	41	64	71	83
Control	204	116.00	80.50	90.00	144.30	126.00					
Cobalt chloride 50 p. p. m.	256	87.30	69.60	80.00	97.50	137.00	−24.34	−13.66	−11.64	−32.43	+ 9.13
Potassium acetate 0.02 *M*	203	121.00	78.25	95.00	95.00	128.00	+ 4.50	− 2.80	+13.81	−34.16	+ 1.58
Ascorbic acid 10 p. p. m.	193	107.50	84.50	116.00	155.80	142.00	− 7.33	+ 4.96	+28.18	+ 7.90	+12.69
Cholesterol 10% oint.	186	79.50	59.50	72.00	107.50	128.00	−31.38	−26.09	−20.44	−25.49	+ 1.58
Petrolatum	44	61.50	64.25	80.00	..	..	−25.94	−44.63	−44.58		
Control (for petrolatum)	38	96.00	120.50	141.80							

[a] Glycoside determinations were made according to a modified Knudson-Dresbach procedure (14).

lower than its controls. Cholesterol ointment, on the other hand, gave yields which were on the average 21.32% higher than its ointment control group. Neither cholesterol ointment nor its ointment control (petrolatum) produced as much total glycoside as did other control groups. However, because of the marked effects of the petrolatum ointment mixture on glycoside yields, it was concluded that the decrease in the case of cholesterol ointment might be caused by its petrolatum base and not by cholesterol. No marked effects were observed on total glycoside formation in leaf material collected from plants treated with potassium acetate in the field. On the other hand, leaf material of ascorbic acid-treated plants gave consistently higher yields of glycoside. Material collected at the end of 64 days showed as much as 28.18% more glycoside than did controls. However, later periodic collections of ascorbic acid-treated plants did not assay significantly higher than controls. It was concluded that ascorbic acid might influence glycoside formation in some degree during early growth. The study was then continued under more carefully controlled hydroponic culture conditions.

Effects Induced During Culture Solution Feedings.—A modified Hoagland culture solution was employed for this study and plants were arranged in 3-L. black culture bottles according to the method of Tsao and Youngken (14). The feedings of chemical substances were commenced in 60- to 70-day-old plants and continued for seven to eight weeks.

The Effects of Cobalt.—From the field study it was observed that toxic responses occurred in digitalis plants which were treated with cobalt chloride in the concentration of 50 parts per million. The following concentrations of cobalt chloride were employed in culture solution; 5, 10, 15, 20, 25, and 50 parts per million (p. p. m.). In the first experiment each group consisted of 10 plants of approximately the same age and size. Digitalis plants which were treated with 50 p. p. m. cobalt chloride in culture solution died at the end of four days. These were immediately harvested. Those treated with 25 p. p. m. cobalt chloride showed brownish-yellow mottled spots and wilting at the end of five days. These were then harvested at the end of seven days. The materials obtained from the above two groups were not enough for glycoside analyses. Plants grown in cobalt chloride in the concentrations of 15 and 20 p. p. m. did not show any growth during two weeks of treatment (see A4, A5, Fig. 1). Following this period they began to wilt. Some of the leaves showed brownish-yellow spots. They were harvested at the end of 14 days. The materials of these two groups were also not sufficient for glycoside analyses. Plants treated with cobalt chloride in concentrations of 5 and 10 p. p. m. showed some inhibition of growth but survived in sufficient numbers for careful growth measurement and glycoside analyses. Some plants treated with 10 p. p. m. cobalt chloride exhibited brownish-yellow spots of a similar nature to those grown with higher concentrations of cobalt. Plants were then collected at the end of 42 days. By this date the average length of blade and petioles of leaves of control plants increased 8.6 cm. (96%), those treated with cobalt chloride (5 p. p. m.) increased 5.6 cm. (62%), and in cobalt chloride (10 p. p. m.) 4.4 cm. (52%). The growth rate of leaves of digitalis grown in cobalt chloride 5 p. p. m. was averagely 34% and in 10 p. p. m., 44% lower than controls (A1, A2, A3, Fig. 1).

The total glycoside yield in digitalis controls and in cobalt chloride 5 and 10 p. p. m. is shown in Table II. Significant differences in total glycoside yields were observed following chemical and pigeon assays of plants treated with cobalt chloride in the concentration of 10 p. p. m. (Table II and Fig. 4). Total glycosides in control plants were 24.75% higher than in plants treated with 10 p. p. m. cobalt chloride by the colorimetric assay and 21.24% higher than the latter by pigeon assay. No significant differences were noted with lower concentrations of cobalt.

In a second experiment a periodic study of glycoside formation in plants grown with 10 p. p. m. cobalt chloride was undertaken. Plants were grown in culture solution for 21 days in order to produce enough leaf material for analyses. At the end of 21 days in solution, 25 plants were harvested from the control group for glycoside analyses. At the same time feedings with 10 p. p. m. cobalt chloride in Hoagland solution were commenced. Control plants were, of course, continued. Twenty plants from each of the treated and control groups were harvested at the end of 7, 14, and 21 days of treatment. At the end of 21 days the growth responses of plants in the two groups showed a significant difference. This result agreed with results of the previous study. From the results of colorimetric and biological analyses there was definite evidence that glycoside formation was significantly inhibited during the first seven days of cobalt feeding. In

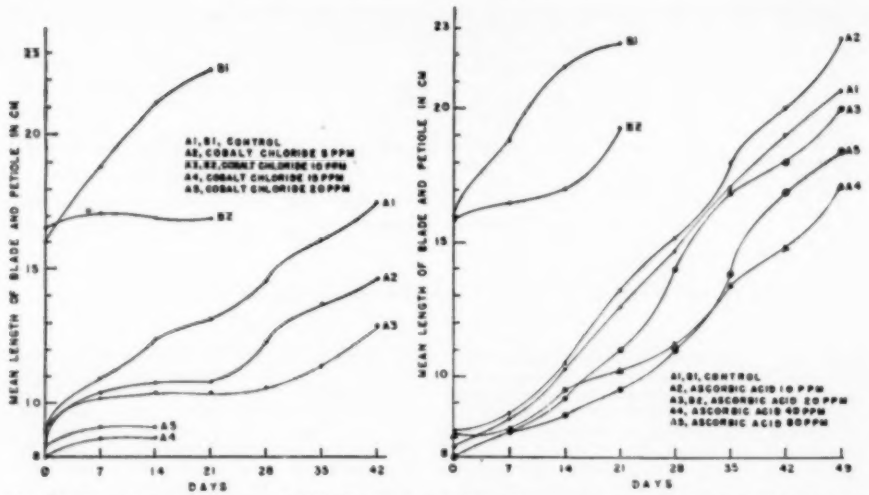

MEAN LENGTH OF BLADE AND PETIOLE IN CM
DAYS
A1, B1, CONTROL
A2, COBALT CHLORIDE 5 PPM
A3, B2, COBALT CHLORIDE 10 PPM
A4, COBALT CHLORIDE 10 PPM
A5, COBALT CHLORIDE 80 PPM
A1, B1, CONTROL
A2, ASCORBIC ACID 10 PPM
A3, B2, ASCORBIC ACID 20 PPM
A4, ASCORBIC ACID 40 PPM
A5, ASCORBIC ACID 80 PPM

Fig. 1.—Growth measurements of digitalis treated with various concentrations of cobalt chloride and ascorbic acid in hydroponic culture.

TABLE II.—RESULTS OF GLYCOSIDE ANALYSES FOLLOWING CHEMICAL FEEDINGS OF DIGITALIS PURPUREA IN CULTURE SOLUTION

Experimental Group	Colorimetric Assay: No. of Plants	Colorimetric Assay: No. of Tests	Colorimetric Assay: Per Cent Potency[a]	Biological Assay: No. of Tests	Biological Assay: Per Cent Potency[a]	Standard Error	"t" Test	Significance of "t" Test
Control	10	4	135.00					
Cobalt chloride 5 p. p. m.	10	4	118.00					
Control	20	4	194.00	6	103.52	±9.70		
Cobalt chloride 10 p. p. m.	20	4	154.00	4	61.17	±5.70	7.200	Less than 0.1%
Control	20	4	181.00	4	98.94	±5.40		
Cobalt chloride 10 p. p. m.	20	4	136.00	4	63.68	±2.00	14.420	Less than 0.1%
Control	20	4	217.00	4	117.21	±7.50		
Cobalt chloride 10 p. p. m.	20	4	128.00	4	71.03	±3.34	11.600	Less than 0.1%
Control	12	4	135.00	4	110.06	±6.63		
Ascorbic acid 20 p. p. m.	12	4	168.00					
Ascorbic acid 40 p. p. m.	11	4	173.00	4	113.14	±9.45	0.585	50-60%[b]
Ascorbic acid 80 p. p. m.	11	4	166.50					
Control	10	4	176.00	4	120.20	±8.65		
Cholesterol 0.5%	10	4	218.00	4	148.59	±9.05	5.000	Less than 1%
Cholesterol 2%	10	4	214.00	4	151.99	±12.00	4.650	Less than 1%
Control	7	4	189.00					
Potassium acetate 0.04 *M*	8	4	177.00					
Sodium acetate 0.04 *M*	8	4	185.00					

[a] Mean per cent potency in terms of U. S. P. Digitalis Reference Standard.
[b] Not significant.

the two analyses the glycoside decrease in treated plants during the first seven days measured as much as 20.36% colorimetrically and 40.92% biologically.

The Effect of Acetate.—From field studies previously described digitalis plants showed no significant toxic responses to as much as 0.02% (0.02 *M*) solution of potassium acetate. Furthermore, there was no apparent influence from these treatments on total glycoside yields. For the solution culture study, potassium and sodium acetates in the concentrations of 0.04 *M* were used. The *p*H of solutions was adjusted by the addition of acetic acid to *p*H 5.1 to 5.2. The same concentrations of potassium and sodium chloride feedings were studied simultaneously with acetate feedings in order that any potassium, sodium, or acetate effects might be separated from those of the corresponding cations and anions. It is an established fact that osmotic pressures such as those due to differences in molar concentrations of solutions may be influencing factors in the ab-

sorption of water by plants (17). Therefore, several different concentrations of Hoagland solutions such as 0.01, 0.04, 0.05, and 0.08 *M* without the addition of acetate were employed in order to examine effects of this potential factor on digitalis growth. The result showed that digitalis plants could survive very well in Hoagland solutions with each of the above molar concentrations and no differences in growth were noted. In one experiment 91 plants of approximately the same size and age (68 days old) were divided into 12 groups, each of which consisted of 6 to 8 plants. These had been grown previously in Hoagland for 35 days in order to produce sufficient leaf growth. One group was then treated with potassium acetate and a second group was treated with sodium acetate. Approximately thirty minutes following the treatment some plants in potassium and sodium acetate began to wilt, and about three hours later all had shown toxic effects. Plants grown in potassium and sodium chloride feedings showed no toxic effects (Fig. 2) Acetate plants recovered about six hours after treatment. All treated and control plants were collected for glycoside analyses twenty-four hours following the treatment.

In a second experiment 107 digitalis seedlings of nearly the same size and age (90 days) were divided into 9 groups, each of which consisted of 10 to 13

Fig. 2.—Growth effects produced by various molar concentrations of potassium and sodium acetates, and potassium and sodium chlorides in *Digitalis purpurea*.
KC4H4—KCl 0.04 *M* in Hoagland solution (0.04 *M*).
KA4H4—Potassium acetate 0.04 *M* in Hoagland solution (0.04 *M*).
H8—Control in Hoagland solution (0.08 *M*).
NA4H4—Sodium acetate 0.04 *M* in Hoagland solution (0.04 *M*).
NC4H4—NaCl 0.04 *M* in Hoagland solution (0.04 *M*).
KC4H1—KCl 0.04 *M* in Hoagland solution (0.01 *M*).
KA4H1—Potassium acetate 0.04 *M* in Hoagland solution (0.01 *M*).
H4—Control in Hoagland solution (0.05 *M*).
Na4H1—Sodium acetate 0.04 *M* in Hoagland solution (0.01 *M*).
NC4H1—NaCl 0.04 *M* in Hoagland solution (0.01 *M*).
Period of treatment—3 hr.

plants. These plants were grown in Hoagland solutions for 20 days before treatments were induced. Plants which were grown in potassium and sodium acetates in 0.04 *M* Hoagland solution with *p*H adjusted to 5.2 began to wilt in forty-five minutes following treatment; those grown in potassium and sodium acetate in 0.01 *M* Hoagland solution with *p*H adjusted to 6.4 showed less toxic responses although a few leaves showed a small degree of wilting (Fig. 2). One-half of the control plants and one-half of those treated with potassium and sodium acetates which showed significant wilting were then harvested for glycoside analyses. Approximately sixty hours after treatment, the remaining plants grown in acetate solution began to wilt again, and wilting persisted. All plants of this experiment were then collected for glycoside determination. The *p*H determinations were made in each case of both fresh and used culture solutions. No change in *p*H was noted during the growth of plants in buffered acetate solutions. The colorimetric analyses of total glycoside did not show any significant difference between digitalis grown in acetates, chlorides, or controls. It was then concluded that acetate in the concentrations employed inhibited the growth response but had no effect on glycoside formation following one to three days of treatment.

The Effect of Ascorbic Acid.—Results from field studies in which digitalis plants were grown with supplementary treatments employing ascorbic acid showed that ascorbic acid did not influence the growth response but that it effected a stimulation of glycoside synthesis to some extent in young plants. In order to determine concentrations of ascorbic acid which could be tolerated by digitalis plants grown in Hoagland solution, it was necessary to employ ascorbic acid solutions covering a wide range of concentrations; 10, 20, 40, and 80 p. p. m. ascorbic acid in Hoagland solution were therefore used and plants were arranged into four groups for each treatment. Each group consisted of 11 to 12 plants of approximately the same age and size. In 80 p. p. m. ascorbic acid solution, 2.5 cc. of 1% sodium hydroxide solution was added to each liter of solution to adjust the *p*H to 5.7. Plants were grown in ascorbic acid solutions in these concentrations for 49 days. During this period growth effects were observed. Concentrations of ascorbic acid in quantities of 20, 40, and 80 p. p. m. showed significant toxic effects in about two weeks (Fig. 1). The growth rate was markedly retarded but no wilting or leaf discoloration was observed. On the other hand, plants treated with lower concentrations showed no significant inhibitory effects. However, because of toxic responses from high concentrations of ascorbic acid the supplementary "treatment" solution was then alternated with culture solution, i. e., plants were grown in ascorbic acid supplementary solution for 14 days, then untreated culture solution for 21 days, followed by "treatment" solutions. At the end of 49 days of treatment, the growth responses of leaves of digitalis plants among controls and ascorbic acid treatments in concentrations of 10 and 20 p. p. m. showed no significant differences, but the responses of leaves of plants grown in concentrations of 40 and 80 p. p. m. showed slower growth (A4, A5, Fig. 1). The average length of blades and petioles of leaves of control plants increased 13.34 cm. (184%), whereas those treated with ascorbic acid in 40 p. p. m. increased 9.30 cm. (120%) and those with 80 p. p. m. had a total increase of 10.60 cm. (136%).

The total glycoside yield of digitalis controls and ascorbic-acid treated groups assayed by the colorimetric and biological methods is shown in Table II. Significant differences in glycoside yields were observed in digitalis plants treated with ascorbic acid in concentrations of 20, 40, and 80 p. p. m. The total glycosides in control plants measured on the average 135.00%, whereas leaf material from digitalis treated with ascorbic acid in concentrations of 20, 40, and 80 p. p. m. analyzed 168.00, 173.00, and 166.50%, respectively. Leaf material of plants treated with 40 p. p. m. ascorbic acid were then also assayed biologically. These results however, showed no significant differences (see Table II and Fig. 4).

In a second experiment 145 digitalis plants of nearly the same size and age were transplanted into culture bottles. The control group consisted of 85 plants, whereas the treated group (ascorbic acid 20 p. p. m.) consisted of 60 plants. These were grown in Hoagland solution for 21 days in order to produce sufficient leaf growth. At the end of 21 days in culture solution, 25 plants were harvested from the control group. On the same day feedings with 20 p. p. m. ascorbic acid for the plants in the treated group were commenced (B1, B2, Fig. 1). Twenty plants from each of the control and treated groups were harvested at the end of 7, 14, and 21 days of treatment. Leaf material was prepared for glycoside analyses at each collection. At the end of 21 days of treatment the growth response of plants in these two groups showed a significant difference. The average length of blade and petiole portions of leaves of control plants increased 6.42 cm. (40.12%) and that in the treated group increased 2.44 cm. (15.36%) (B1, B2, Fig. 1). This growth response was observed to be different from the previous study during which younger plants were employed. The difference was thought to be due to the age factor. Plants in the previous experiment were 56 days old, whereas those in this study were 106 days old.

From results of colorimetric and biological analyses of the latter experiment, there appeared to be no significant differences in glycoside production. This finding was inconsistent with that of the first experiment in which younger plants were studied and in which the glycoside yields following ascorbic acid treatment were generally higher than in controls. This difference was thought to be due to differences in the age of digitalis studied in the two experiments. Results indicated that the glycoside formation in younger, actively growing digitalis plants is significantly influenced by ascorbic acid feedings. As plants become older (for example, in the second experiment plants were 50 days older than in the first), the effects from ascorbic acid feedings on glycoside production are generally insignificant.

Tests were then made of ascorbic acid and dehydroxyascorbic acid in order to ascertain what the true results of colorimetric tests would be with these compounds under the same conditions. Results were negative.

The Effect of Cholesterol.—Results from field studies indicated that the application of cholesterol

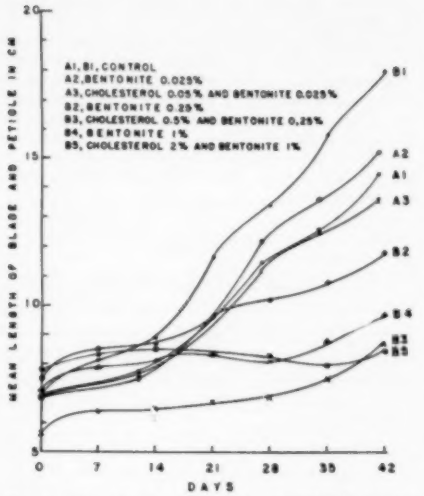


Fig. 3.—Growth measurements of ditigalis treated with various concentrations of cholesterol suspension.

in ointment form was not a suitable preparation for the growth study and glycoside response of digitalis because of interfering effects produced by the ointment base. It was deemed necessary, therefore, to examine the effect of cholesterol under more carefully controlled conditions such as culture solution. Because of the insolubility of cholesterol in culture solution, a cholesterol suspension was used with bentonite as the suspending agent. The ratio of cholesterol to bentonite was 2 to 1. Suspensions of bentonite in culture solution without cholesterol were also employed as controls. Plants were grown in each of these solutions for one-week periods, after which they were placed into Hoagland's solution for weekly intervals. By alternating treatment, e. g., by using suspensions for one week, followed by culture solution the next week, etc., plants were present in cholesterol suspension for three weeks. This study was divided into two experiments in which varying concentrations of cholesterol were used for feeding purposes.

In the first experiment, 58 plants of approximately the same size and age were arranged in culture bottles. Cholesterol 0.05% and bentonite 0.025% were used for the treated group. The control plants were grown in a 0.025% suspension of bentonite in distilled water. At the end of 41 days, leaves were collected and prepared for glycoside analyses. Following 41 days of treatment, leaf growth of cholesterol-treated and bentonite control groups showed no significant differences (A1, A2, A3, in Fig. 3). The total glycoside yield in digitalis controls and those treated with these concentrations of cholesterol showed no significant differences when assayed by colorimetric and biological methods.

In a second experiment, cholesterol suspensions of higher concentrations, i. e., 0.5 and 2%, were employed. These concentrations were employed in the same manner as above. Fifty plants of nearly the same size and age were divided into five groups: one group containing distilled water alternated with Hoagland solution, two groups each consisting of bentonite 0.25 and 1% alternated with Hoagland solution, and two groups each consisting of cholesterol 0.5 and 2% alternated with Hoagland solution. The treatment was carried on for 42 days. The growth response showed significant differences (B1–5, Fig. 3). The average length of blades and petioles of control plants increased 10.94 cm. (157%), those treated with 0.25% bentonite "controls" increased 4.31 cm. (57%), and with 1% bentonite control the growth of leaves was increased 2.53 cm. (34%). Leaves of plants treated with cholesterol added to bentonite in concentration of 0.5% increased 3.15 cm. (55%) and with 2% the average increase of leaf blades and petioles was 0.75 cm. (10%).

The total glycoside yields were assayed by both colorimetric and biological methods. Significant differences were observed in digitalis plants grown with cholesterol of both 0.5 and 2% concentrations (Table II and Fig. 4). Results of colorimetric analyses indicated that the total glycoside in culture solution controls measured averagely 176%; whereas the leaf material from digitalis treated with cholesterol in concentrations of 0.5 and 2% analyzed 218% (+23.86%) and 214% (+21.59%) total glycosides, respectively. Plants treated with bentonite alone in concentrations of 0.25 and 1% yielded approximately 186.00% (+5.68%) and 209.00% (+18.73%) total glycosides. The biological analyses of controls and cholesterol-treated groups showed that digitalis plants treated with cholesterol suspensions of 0.5 and 2% gave, respectively, 23.62 and 26.45% higher glycoside potency (Fig. 4). It was concluded that cholesterol in these concentrations exerted a stimulating response on digitalis glycoside production.

DISCUSSION

When varying concentrations of chemical compounds such as cobalt chloride, ascorbic acid, and cholesterol were fed to digitalis plants growing in the field and in hydroponic culture, a significant effect was observed on growth and total glycoside production in the leaves. In general, these compounds, in the concentrations employed, inhibited growth. Cobalt chloride in the concentration of 10 p. p. m. produced a significant decrease in total glycoside yields. In view of the reports of Ahmad, *et al.* (1), who have proposed that cobalt exerts profound interference with carbohydrate metabolism and respiration in some plants, it was concluded that this compound may have inhibited glycoside synthesis in digitalis in some manner through an arrest of carbohydrate function.

Potassium acetate, on the other hand, in the concentration of 0.02 *M*, did not give any significant difference in both growth and glycoside response in digitalis plants growing under field conditions. However, potassium and sodium acetates in the concentrations of 0.04 *M* produced a significant toxic effect on growth of digitalis in hydroponic culture. It was concluded that the toxicity was due to the influence of concentrations of the acetate ions, and not to that of cations or molarity, for sodium and potassium chloride in the same concen-

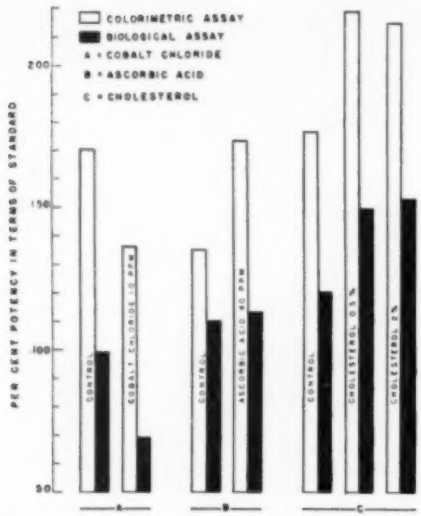


Fig. 4.—Bar graph showing response of chemical growth regulators on glycoside formation in digitalis.

trations as acetates did not show any toxic effect on digitalis growth. Furthermore, plants fed with 0.05 and 0.08 *M* Hoagland solution which had the same molar concentrations as acetate solutions also did not produce any toxic effect. There was evidence, therefore, that acetate ions in the same amounts employed interfered directly with digitalis growth and that high titers of this compound or its product, acetic acid, did not augment glycoside production in the plant.

Ascorbic acid and cholesterol feedings resulted in increased glycoside production but both compounds inhibited growth in varying degrees. The true effects of cholesterol on plant growth are rather difficult to interpret because of the need to employ this water-insoluble compound in a suspending agent for adequate feeding purposes.

Glycoside biosynthesis following the use of ascorbic acid in concentrations of 20 and 40 p. p. m. was found to be increased significantly when leaf material was assayed according to the conventional colorimetric method. This was true in field and culture solution studies in the case of young 56-day-old plants. It was not apparent in older plants (106 days old). However, biological analyses during which the U. S. P. pigeon method was employed failed to reveal any significant increase in cardiotonic glycoside production at any age. Therefore, it was concluded that ascorbic acid may have exerted its greatest influence on the stimulation of glycoside components other than certain physiologically active compounds such as would be sensitive to biological methods for evaluation. Furthermore, this influence is perceptible only in active and younger plants.

There has been no attempt made during this study to establish the means by which the glycoside stimulating effect produced by cholesterol did occur. However, on the basis of this stimulating influence it is suggested that cholesterol affected biosynthesis *per se*, or it may have been degradated by chemical forces which are the function of the plant to form simpler compounds, which in turn stimulated glycoside synthesis.

SUMMARY

1. Various concentrations of cobalt chloride, ascorbic acid, potassium acetate, and cholesterol were fed to digitalis plants growing in the field and in hydroponic culture solutions. Certain inhibitory growth responses are described following the use of these compounds during several weeks of treatment.

2. Cobalt chloride in concentration of 10 p. p. m. produced marked inhibition of total glycoside production and this was evident early during the feeding experiment.

3. No significant effects were observed on the biosynthesis of digitalis glycosides following feedings with 0.02 and 0.04 *M* (0.2 and 0.4 per cent) solutions of potassium or sodium acetates or their chlorides.

4. Ascorbic acid in concentrations of 20 and 40 p. p. m. stimulated an increase in total glycosides only in young, actively growing plants.

5. Cholesterol in concentrations of 0.5 and 2 per cent stimulated glycoside biosynthesis during the 21 days of feeding with this compound.

REFERENCES

(1) Ahmad, B., *et al.*, *Ann. Biochem. Exptl. Med.*, **6**, 29 (1946).
(2) Schwarrer, K., and Schropp, W., *Z. Pflanzenernähr. Dungung. u. Bodenk.*, **A31**, 94(1933).
(3) Krotkov, G., and Barker, H. A., *Am. J. Botany*, **35**, 12(1948).
(4) Mapson, L. W., and Cruickshank, E. M., *Biochem. J.*, **41**, 197(1947).
(5) Sugawara, T., *Japan. J. Botany*, **11**, 344(1941).
(6) Hausen, S., *Nature*, **136**, 516(1935).
(7) Havas, L., *ibid.*, **137**, 435(1935).
(8) Povolatskaya, K. L., *Bull. Applied Botany Genetics Plant Breeding. Leningrad*, Suppl. No. 84, (1937).
(9) Ray, S. N., *Biochem. J.*, **28**, 996(1934).
(10) Sideris, C. P., *et al.*, *Plant Physiol.*, **24**, 265(1949).
(11) Virtanen, A. I., *Nature*, **137**, 779(1936).
(12) Much, V. H., Nyren, T., and Schubert, J., *Zentr. Bakt. Parasitenk., I Abt.*, **116**, 1(1930).
(13) Brady, R. O., and Gurin, S., *J. Biol. Chem.*, **187**, 589(1950).
(14) Tsao, D. P. N., and Youngken, H. W., Jr., THIS JOURNAL, **41**, 19(1952).
(15) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950, p. 179.
(16) Hall, M. F., "The Presentation of Medical Statistics," Overbeck, Ann Arbor, 1946, p. 33.
(17) Meyer, B. S., and Anderson, D. B., "Plant Physiology," D. Van Nostrand, New York, 1939, p. 283.

Resin Sikkimensis. I. Sikkimotoxin: a Lactone from *Podophyllum sikkimensis* R. Chatterjee *et* Mukerjee*

By R. CHATTERJEE and S. C. CHAKRAVARTI†

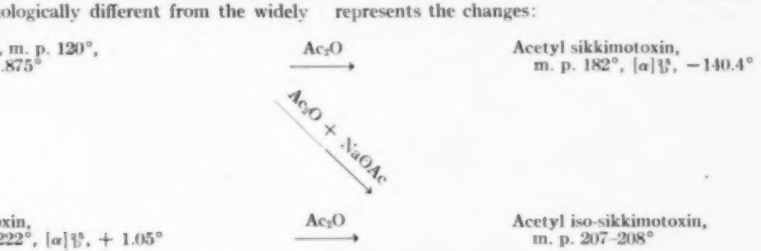
The lactone, sikkimotoxin, is analogous to podophyllotoxin, a component of *P. peltatum* L. and *P. emodi* Wall. Based on the action of some oxidizing and reducing agents, acetic anhydride and acetyl chloride, a tentative formula (I) has been suggested for it.

SOME of the specimens of *Podophyllum* collected from the Sikkim Himalayas appeared to be morphologically different from the widely known Indian species *P. emodi* Wall., and these specimens were found to represent a new species which has been called *P. sikkimensis* (1). One of the components of the rhizomes of this new species is a colorless crystalline substance, m. p. 120° with effervescence, which has been called sikkimotoxin (2) and is represented by the molecular formula $C_{23}H_{26}O_8$. This appears to be similar to podophyllotoxin, $C_{22}H_{22}O_8$, a component of the rhizomes of the American species *P. peltatum* L. and the Indian species *P. emodi* Wall.

Sikkimotoxin contains five methoxyl groups. It forms a monoacetyl derivative, thus showing the presence of one hydroxyl group which is alcoholic in nature, for the toxin does not react with diazomethane. It does not react with sodium bicarbonate solution. It shows an equivalent of one carboxyl group when titrated with sodium hydroxide solution, indicating the presence of one lactone ring in its molecule. All of the eight oxygen atoms in one molecule of sikkimotoxin are thus accounted for, and the formula of sikkimotoxin is extended to:

$$C_{16}H_8\begin{cases}-5\ \text{OMe}\\-1\ \text{OH (alcoholic)}\\-\text{CO—O—}\end{cases}$$

Sikkimotoxin forms iso-sikkimotoxin, m. p. 220-222°, on warming with ethanolic solution of sodium acetate, aqueous sodium carbonate, sodium hydroxide solution, or with pyridine solution. The acetyl derivative of sikkimotoxin behaves in a similar manner and is transformed to the acetyl derivative of the isomer, on warming with sodium acetate. The following scheme represents the changes:


Sikkimotoxin, m. p. 120°, [α]$_D^{35}$, −91.875°
Ac$_2$O
Acetyl sikkimotoxin, m. p. 182°, [α]$_D^{35}$, −140.4°
Ac$_2$O + NaOAc
iso-Sikkimotoxin, m. p. 220–222°, [α]$_D^{35}$, + 1.05°
Ac$_2$O
Acetyl iso-sikkimotoxin, m. p. 207–208°

Sikkimotoxin reacts with acetyl chloride to produce monochloro-sikkimotoxin, which indicates that its alcoholic hydroxyl group is secondary in nature. iso-Sikkimotoxin does not react with acetyl chloride. Sikkimotoxin furnishes 2-methylnaphthalene (VI) on zinc dust distillation in a current of hydrogen. iso-Sikkimotoxin produces 6, 7-dihydroxy-2-methylnaphthalene-3-carboxylic acid (VII, R = H) on treatment with hydriodic acid. The reactions in both cases show the presence of substituents at 2 and 3. The formation of the methyl group in position 2, in both cases, indicates, in all probability, the presence of a primary alcoholic group at 2, in sikkimotoxin as well as iso-sikkimotoxin. This group forms lactone with the carboxyl group at 3, for a potential carboxyl group must be present in that position in iso-sikkimotoxin which is prominent in VII.

When oxidized with potassium permanganate and alkali, sikkimotoxin degrades to ω-(3′,4′,-5′ - trimethoxyphenyl) - 4,5 - dimethoxyphthalide (IV) and 4,5,3′,4′,5′-pentamethoxy-2-benzoylbenzoic acid (V). The phthalide (IV), in turn, is oxidized to V with potassium ferricyanide and alkali. This establishes the structure of the phthalide (IV), for in the presence of alkali the phthalide ring opens and leaves the liberated =CHOH group to oxidize to the —CO group, as

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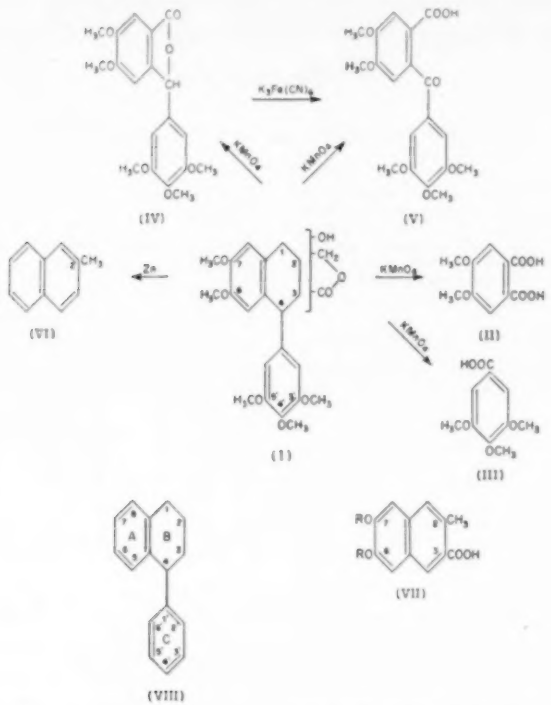
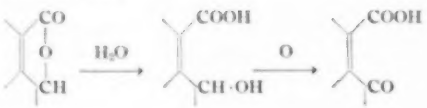


shown below:



(IVa) (Va)

The production of IV and V also proves that sikkimotoxin contains the benzene nucleus linked to the naphthalene moiety as in VIII.

Sikkimotoxin also yields *meta*-hemipinic acid (II) and trimethylgallic acid (III), on permanganate oxidation, to prove that two of its five methoxyl groups are located at 6 and 7 of ring A, and the remaining three at 3′, 4′, and 5′ of ring C.

The ring B contains the substituents, the lactone at 2, 3, and the secondary alcoholic group and the ring C in positions 1 and 4 or vice versa. It is less stable than the rings A and C; its instability can be explained if it is considered to be hydrogenated, which is probably the case, for the production of the phthalide (IV), as well as the presence of a secondary alcoholic group, points to the presence of at least partial, if not full, hydrogenation of ring C. On the basis of the experiments made so far, a tentative structure (I) can be suggested for sikkimotoxin.

Further work on the elucidation of the structures of sikkimotoxin and iso-sikkimotoxin is in progress.

EXPERIMENTAL

Isolation of Resin Sikkimensis.—Air-dried, powdered (60-mesh) roots and rhizomes (1 Kg.) of *P. sikkimensis* were extracted continuously with ethanol (90%) and the ethanolic extract was evaporated to a syrupy consistency and poured with constant stirring into hydrochloric acid solution (1%, 250 cc.) cooled to 5°; a flocculent precipitate of the resin sikkimensis was obtained which was kept cool for two hours, filtered, washed with the acid, and dried. Yield, 75 Gm.

Isolation of Sikkimotoxin from Resin Sikkimensis.—The brown resin (75 Gm.) was extracted with chloroform, and the sticky brown extract (40 Gm.) was dissolved in ethanol (50 cc.). The solution was mixed with well-cooled benzene (50 cc.) to precipitate white sikkimotoxin (35 Gm.), m. p. 120° with effervescence.

Anal.—Calcd. for $C_{22}H_{28}O_9$: C, 64.2; H, 6.05; OMe, 36.05. Found: C, 64.1; H, 5.8; OMe, 35.92.

Sikkimotoxin is highly soluble in ethanol, methanol, and chloroform; moderately soluble in isobutyl alcohol; sparingly soluble in cold benzene,

though more soluble in a large volume of boiling benzene; insoluble in water, petroleum ether (b. p. 60–80°), and sulfuric ether. It is levorotatory, $[\alpha]_D^{25°}$ -91.875.

Zinc Dust Distillation of Sikkimotoxin.—Sikkimotoxin (3 Gm.) was intimately mixed with zinc dust (10 Gm.) and heated to redness in a tube in a current of hydrogen gas. A small quantity of volatile product which collected in the cooler part of the tube and had the odor of naphthalene was taken up in dry ether and the oily residue on removal of ether was distilled at 120° at 8 mm. pressure, and was mixed with ethanolic solution of picric acid to deposit crystals of a picrate, m. p. 114°. The mixed melting point of an authentic sample of 2-methyl naphthalene (VI) was 114°.

Anal.—Calcd. for $C_{17}H_{13}O_7N_3$: C, 54.97; H, 3.50. Found: C, 54.50; H, 3.71.

Action of Hydriodic Acid upon iso-Sikkimotoxin: Identification of 6,7-Dimethoxy-2-methylnaphthalene-3-carboxylic Acid (VII, R = Me) (3).—Iso-Sikkimotoxin (5 Gm.) in acetic acid (50 cc.) was boiled with hydriodic acid (sp. gr. 1.7; 40 cc.) for one-half hour and, on cooling, some muddy-brown crystals separated from the brown-colored solution. The mixture was treated with sulfur dioxide, made basic with sodium hydroxide, and shaken with an excess of dimethyl sulfate. The precipitated methoxy compound was filtered and boiled with sodium hydroxide solution, cooled, and then treated with hydrochloric acid to precipitate the methoxy derivative of an acid identical with 6,7-dimethoxy-2-methylnaphthalene-3-carboxylic acid. The acid was crystallized from acetic acid as colorless needles, m. p. 223–225°.

Anal.—Calcd. for $C_{14}H_{14}O_4$: C, 68.29; H, 5.70; OMe, 25.2. Found: C, 67.51; H, 5.71; OMe, 25.3.

Oxidation of Sikkimotoxin with Potassium Permanganate: Isolation of *meta*-Hemipinic Acid (II) and Trimethylgallic Acid (III).—A solution of sikkimotoxin (4 Gm.) in potassium hydroxide solution (5%, 200 cc.) was heated to boiling and was treated at that temperature with potassium permanganate solution (5%, 20 cc. at a time) with occasional vigorous shaking until the pink color disappeared. The decolorization of the permanganate solution was very rapid at first and grew slower when about 400 cc. of the permanganate solution (equivalent to 0.19 gram atom of oxygen) was added. After the addition of 100 cc. more of the permanganate solution (equivalent to 0.0475 gram atom of oxygen) the pink color persisted and the hot solution was allowed to stand for three hours, by which time the precipitated manganese dioxide settled and was filtered off. The residue of manganese dioxide was thoroughly washed with water and the washings were added to the main bulk of the filtrate. The pale pink color of the filtrate was discharged by adding sulfur dioxide and the filtrate was then cooled in ice and was made acid to congo red with hydrochloric acid. A white solid which separated from the solution was filtered, washed with water, and the washings were bulked with the filtrate.

(*A*) *Treatment of the White Solid.*—The solution of the solid in ether (100 cc.) was washed twice with water to remove any trace of mineral acid and then dried. On removal of ether a pale yellow solid was obtained which dissolved in warm dilute ammonium hydroxide solution (1–4, 20 cc.). The ammoniacal solution was almost neutralized with hydrochloric acid solution (5%), and the slightly ammoniacal solution was then warmed and treated with a solution of 5% calcium chloride to turbidity. The mixture was allowed to stand for one hour and the small quantity of the white precipitate of calcium oxalate was removed. The filtrate was ice-cooled and treated with 10% hydrochloric acid solution to obtain a white precipitate (80 mg.), which was filtered.

The white precipitate dissolved in aqueous sodium bicarbonate solution with slight effervescence. It produced a fluorescent compound on heating with resorcinol and concentrated sulfuric acid. It was dissolved in ethanol and was filtered after Norit treatment. The filtrate was diluted with water to turbidity and was left in the refrigerator to yield pale-ochre-colored crystalline needles. The crystals were separated from the mother liquor, which on concentration furnished a further crop of crystals. Total yield, 50 mg., m. p. 202–204° with vigorous effervescence on slow heating. On rapid heating, however, the crystals melted at 189–191° with decomposition.

Anal.—Calcd. for $C_{10}H_{10}O_6$: C, 53.1; H, 4.43. Found: C, 52.9; H, 4.30.

The combustion analysis and the properties of the crystals indicated them to be of one of the hemipinic acids, for Fargher and Perkin (4) mentioned that *meta*-hemipinic acid melted either at 202–204° with effervescence, or at 189–190° with decomposition, depending upon the rate of heating. Adams and Butterbaugh (5) recorded the melting point of both synthetic *meta*-hemipinic acid and that obtained from apogossypol as 190–191°.

Ethylimide of meta-Hemipinic Acid.—This derivative of the acid obtained was prepared according to Liebermann (6) by dissolving the acid in an aqueous solution of 30% ethylamine and evaporating the solution completely to dryness. The residue was heated by a free flame and then crystallized from boiling methanol as colorless needles, m. p. 230–231°. There was no depression in melting point on admixture with an authentic sample of *meta*-hemipinethylimide, m. p. 230–231°.

Anal.—Calcd. for $C_{12}H_{13}O_4N$: N, 5.96. Found: N, 5.63.

The filtrate was extracted exhaustively with ether (300 cc. in three installments) and, on removal of the solvent, the ether layer furnished a small quantity of pale yellow solid (30 mg.) which was insoluble in cold water but was soluble in a large volume of boiling water. The solid was then crystallized from a large volume of boiling water (charcoal) to obtain colorless needles, m. p. 160–163°. Further crystallization from boiling water raised the melting point to 167–168°. The crystals did not depress the melting point of an authentic sample of trimethylgallic acid, m. p. 167–168°, prepared from gallic acid and dimethyl sulfate (7).

(*B*) *Treatment of the Aqueous Layer.*—The yellow-colored aqueous layer was extracted exhaustively with ether (800 cc. in four installments); on removing the ether from the ether solution, a residue was obtained as a yellow paste which almost solidified on cooling in ice, and was dried *in vacuo*. The solid was dissolved in ammonia (1–4, 20 cc.) and the solution was almost neutralized; the slightly alkaline solution was boiled and finally treated with a solution of

5% calcium chloride to obtain a crystalline white precipitate of calcium oxalate which was removed. When the filtrate was cooled and acidified, a small quantity of a yellow-colored solid separated which was filtered and dissolved in the least quantity of ethanol, treated with animal charcoal, filtered, and the filtrate was diluted with water and was left to obtain crystalline needles, m. p. 181° with effervescence, on rapid heating. The crystals were identified as *meta*-hemipinic acid.

Anal.—Calcd. for $C_{10}H_{10}O_6$: C, 53.1; H, 4.43. Found: C, 52.8; H, 4.6.

The filtrate from this *meta*-hemipinic acid was extracted with ether and, on evaporation of the solvent from the ethereal extract, a pale yellow oil was obtained which was solidified on cooling in ice, but the amount was too small for further examination.

Oxidation of Sikkimotoxin with Potassium Permanganate: Isolation of ω-(3′, 4′,5′-Trimethoxyphenyl)-4,5-dimethoxyphthalide (IV) and 4,5,3′,4′,5′-Pentamethoxy-2-benzoyl Benzoic Acid (V).—Sikkimotoxin (2 Gm.) was dissolved in *N* sodium hydroxide solution (20 cc.) at 50–55° and was treated with 4% potassium permanganate solution, 20 cc. at a time, after waiting for the pink color of the solution to be discharged after each addition. After the addition of 175 cc. (equivalent to .0665 gram atom of oxygen) of permanganate solution, the color of the mixture remained permanently pink and the mixture was cooled and treated with sulfur dioxide to dissolve the precipitate manganese dioxide and discharge the pink color. The colorless solution thus obtained was ice-cooled and acidified to obtain a white solid. The mixture was allowed to stand in an ice bath for an hour and then was exhaustively extracted with 800 cc. of ether. On evaporation of the ethereal solution a white solid residue was obtained which was repeatedly extracted with warm aqueous sodium carbonate (10%). A portion of the residue went in solution, which was filtered.

(*A*) *The Filtrate.*—The filtrate (130 cc.) was cooled and acidified to obtain a flocculent precipitate, which was taken up in ether. The ether layer yielded a solid residue (0.12 Gm.) which was soluble in sodium bicarbonate solution (10%). It was crystallized from a large volume of boiling water (charcoal), and colorless needles (80 mg.), m. p. 207–209°, were obtained. On further crystallization from boiling water and from dilute ethanol the melting point rose to 210–211°.

Anal.—Calcd. for $C_{19}H_{20}O_6$: C, 60.63; H, 5.32. Found: C, 60.61; H, 5.32.

The combustion analysis and the behavior of the crystals indicate them to be of the keto acid (V), which has been established subsequently by converting the phthalide (IV) to the keto acid (V).

(*B*) *The Pale Yellow Residue.*—The residue (0.25 Gm.) crystallized from dilute ethanol as colorless needles, m. p. 205–207°. The crystals are insoluble in 10% sodium bicarbonate solution and sodium carbonate solution. They dissolve readily in warm sodium hydroxide solution (10%), indicating their lactonic character. The combustion analysis indicates further that they are the phthalide (IV).

Anal.—Calcd. for $C_{18}H_{20}O_7 \cdot {}^1/_2H_2O$: C, 61.77; H, 5.73. Found: C, 61.44, 61.74; H, 5.70, 5.54.

Oxidation of ω-(3′,4′,5′-Trimethoxyphenyl)-4,5-dimethoxy Phthalide (IV) with Potassium Ferricyanide: Formation of 4,5,3′,4′,5′-Pentamethoxy-2-benzoyl Benzoic Acid (V).—The phthalide (0.1 Gm.) in 10% potassium hydroxide solution at 60–65° was treated with potassium ferricyanide solution (6%, 5 cc.) added to the solution of the phthalide in small portions with vigorous shaking for ten minutes. This was followed by the addition of potassium hydroxide solution (1.5%, 10 cc.) and subsequently potassium ferricyanide solution (6%, 5 cc.) with rapid shaking. The process of alternate addition of alkali and the ferricyanide solution was repeated until 50 cc. of alkali and 50 cc. of the ferricyanide solution were added. The mixture was then cooled and acidified to precipitate a white solid which was extracted with ether. The solid residue left on removal of ether was treated with warm aqueous sodium carbonate solution (10%, 30 cc.), and when a part of it (0.04 Gm.) remained undissolved, it was filtered. It was found to be unreacted phthalide (IV), for on purification it melted at 205–207° and did not depress the melting point of the phthalide (IV).

The alkaline filtrate was cooled and treated with concentrated hydrochloric acid to yield a voluminous precipitate (40 mg.) which was allowed to stand for one hour and then filtered and crystallized from boiling water as needles, m. p. 207–209°. Repeated crystallization raised the melting point to 209–211°, no depression in the melting point (210–211°) of the keto acid (V).

Anal.—Calcd. for $C_{19}H_{20}O_8$: C, 60.63; H, 5.32. Found: C, 60.6; H, 5.3.

Attempted Oxidation of Sikkimotoxin with Hydrogen Peroxide.—To sikkimotoxin (1 Gm.) in potassium hydroxide solution (5%, 20 cc.) at 60–65°, hydrogen peroxide (12 vols., 125 cc.) was added dropwise with constant shaking. The solution was kept overnight in the refrigerator and was then boiled to decompose the excess of hydrogen peroxide. It was subsequently cooled and treated with dilute hydrochloric acid (5%) to yield a white solid; the mixture was then extracted with ether to obtain a highly crystalline solid (0.9 Gm.) on removal of ether. The solid was triturated with aqueous sodium bicarbonate solution (5%, 20 cc.) and no surprising results were obtained on further treatment of the solution. The unreacted solid was purified by repeated crystallization from the ethanol-ether mixture as colorless needles, m. p. 220–221°.

Anal.—Calcd. for $C_{29}H_{28}O_8$: C, 64.2; H, 6.05. Found: C, 63.98; H, 5.9. $[\alpha]_D^{25°} + 1°.05$ (acetone).

The combustion analysis and the specific rotation indicate the solid to be an isomer of sikkimotoxin which has been called iso-sikkimotoxin.

Formation of iso-Sikkimotoxin.—

By Sodium Carbonate.—Sikkimotoxin (0.5 Gm.) was boiled with aqueous sodium carbonate (2 *N*, 50 cc.) for one-half hour, cooled, and acidified with hydrochloric acid to produce a white solid which was kept overnight in the refrigerator. The solid was then filtered and washed with ice-cold water until free from chloride. The crude solid (0.3 Gm.) was purified as usual by repeated crystallization from ethanol as white needles, m. p. 220–222°.

Anal.—Calcd. for $C_{29}H_{28}O_8$: C, 64.2; H, 6.05. Found: C, 63.99; H, 5.9. $[\alpha]_D^{25°} + 1°$ (acetone).

By Sodium Hydroxide.—Sikkimotoxin (2 Gm.) was heated for one-half hour with *N* sodium hydroxide solution (100 cc.) and then carefully cooled in an ice bath and acidified with hydrochloric acid.

A white solid was precipitated, which was filtered and washed as before. The crude solid (1.2 Gm.) was purified as usual as white needles, m. p. 220–222° (yield, 1.0 Gm.). There was no depression in melting point when this solid was mixed with the previous samples of iso-sikkimotoxin. $[\alpha]_D^{25°}$ + 1°.05 (acetone).

By Sodium Acetate.—Sikkimotoxin (0.1 Gm.) in the least quantity of ethanol was refluxed for twenty-four hours with an ethanolic solution of freshly fused anhydrous sodium acetate (0.1 Gm.). On cooling, a small quantity of colorless needles separated and the solution yielded a further crop of crystals on concentration *in vacuo*. Yield, .04 Gm., m. p. 220–221°; no depression in melting point on admixture with the previous samples.

By Pyridine.—Sikkimotoxin (0.5 Gm.) in methanol (7 cc.) and water (3 cc.) was warmed with pyridine (two drops), and colorless needles of iso-sikkimotoxin then separated, which were filtered, washed, and crystallized from methanol, m. p. 220°: there was no depression in melting point when mixed with iso-sikkimotoxin prepared by the action of sodium hydroxide, sodium carbonate, or sodium acetate.

Anal.—Calcd. for $C_{23}H_{28}O_8$: C, 64.2; H, 6.05; OMe, 36.05. Found: C, 63.98; H, 5.9; OMe, 35.7.

Action of Acetic Anhydride on Sikkimotoxin.—Sikkimotoxin (0.5 Gm.) was heated with 6 cc. of redistilled acetic anhydride for one hour and the clear solution was poured in ice-cold water (120 cc.), whereupon a part of the reaction product solidified and the major portion stayed as an oil which solidified on cooling in a refrigerator for two days. The crude solid (m. p. 170–175°; 0.52 Gm.) of monoacetyl sikkimotoxin crystallized as colorless needles from ethanol. Yield, 0.455 Gm., m. p. 180–182°.

Anal.—Calcd. for $C_{25}H_{30}O_9$: C, 63.55; H, 5.97. Found: C, 63.45; H, 6.01; $[\alpha]_D^{25}$ − 140.4° (chloroform).

Action of Acetic Anhydride on iso-Sikkimotoxin.—iso-Sikkimotoxin (0.5 Gm.) was heated with acetic anhydride (6 cc.) and treated as usual to obtain a solid (0.5 Gm.), monoacetyl iso-sikkimotoxin, which crystallized from ethanol as prisms, m. p. 207–208°.

Anal.—Calcd. for $C_{25}H_{30}O_9$: C, 63.55; H, 5.97. Found: C, 63.16; H, 5.38.

Action of Acetic Anhydride and Sodium Acetate on Sikkimotoxin.—Sikkimotoxin (0.5 Gm.) was heated with freshly fused anhydrous sodium acetate (0.5 Gm.) and redistilled acetic anhydride (7 cc.) for one hour and treated as usual to obtain a solid which crystallized from ethanol as prisms (0.45 Gm.), m. p. 207–208°. The crystals did not depress the melting point when mixed with the monoacetate of iso-sikkimotoxin prepared previously.

Action of Acetic Anhydride and Sodium Acetate on iso-Sikkimotoxin.—iso-Sikkimotoxin (0.5 Gm.) was heated, as in the previous case of sikkimotoxin, with freshly fused anhydrous sodium acetate (0.5 Gm.) and redistilled acetic anhydride (7 cc.) and worked up in like manner to yield the same monoacetate of iso-sikkimotoxin (yield, 0.45 Gm.; m. p. 207–208°.)

Action of Sodium Acetate on Monoacetyl Sikkimotoxin.—Sikkimotoxin monoacetate (0.5 Gm.) in ethanol was refluxed with fused sodium acetate (0.3 Gm.) for eight hours; on cooling, some crystals separated which were filtered, and the mother liquor yielded a further quantity of crystals on concentration *in vacuo*. Total yield, 0.4 Gm.; m. p. 207–208°. There was no depression in melting point when mixed with a sample of the monoacetate of iso-sikkimotoxin prepared previously.

Action of Acetyl Chloride on Sikkimotoxin.—A clear solution of well-dried sikkimotoxin (2 Gm.) in freshly distilled acetyl chloride (3 cc.) was heated under reflux for four hours during which time the color of the solution became pale yellow. The excess acetyl chloride was removed by suction to obtain a vitreous solid which crystallized with difficulty from a mixture of benzene and petroleum ether, b. p. 60–80°, as well-defined prismatic needles melting sharply at 196–197° with effervescence.

Anal.—Calcd. for $C_{23}H_{29}O_7Cl$: C, 61.53, H, 5.57; Cl. 7.9. Found: C, 60.91; H, 5.40; Cl, 8.1.

Action of Acetyl Chloride on iso-Sikkimotoxin.—iso-Sikkimotoxin (1.5 Gm.) was heated in a similar manner with acetyl chloride (7 cc.) to isolate the unchanged compound.

SUMMARY

1. Sikkimotoxin, $C_{23}H_{28}O_8$, a new lactone of the tentative structure I, is obtained from the rhizomes of *Podophyllum sikkimensis* R. Chatterjee *et* Mukerjee.

2. It contains five methoxyl groups, one secondary alcoholic group, and one lactonic group.

3. Sikkimotoxin and acetyl sikkimotoxin change to iso-sikkimotoxin and acetyl iso-sikkimotoxin by the action of ethanolic sodium acetate.

4. Sikkimotoxin yields 2-methyl naphthalene (VI) on zinc dust distillation, and iso-sikkimotoxin furnishes 6, 7-dihydroxy-2-methylnaphthalene-3-carboxylic acid (VII, R = H) on reduction with hydriodic acid.

5. On oxidation with potassium permanganate, sikkimotoxin produces ω-(3′, 4′, 5′-trimethoxyphenyl)-4, 5-dimethoxyphthalide (IV), 4, 5, 3′, 4′, 5′-pentamethoxy-2-benzoyl benzoic acid (V), trimethylgallic acid (III), and *meta*-hemipinic acid (II).

REFERENCES

(1) Chatterjee, R., and Mukerjee, S. K., *Indian J. Physiol. Allied Sci.*, **4**, 7(1950).
(2) Chatterjee, R., and Dutta, D. K., *ibid.*, **4**, 61(1950).
(3) Borsche, W., and Niemann, J., *Ann.*, **499**, 59(1932).
(4) Fargher, R. G., and Perkin, W. H., *J. Chem. Soc.*, **119**, 1724(1921).
(5) Adams, R., and Butterbaugh, D. J., *J. Am. Chem. Soc.*, **60**, 2174(1938).
(6) Liebermann, C., *Ber.*, **19**, 2275(1886).
(7) Gilman, H., and Blatt, A. H., "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, 1947, p. 537.

The Action of Riboflavin on Folic Acid*,†

By STANLEY SCHEINDLIN, ARTHUR LEE, and IVOR GRIFFITH

Folic acid undergoes oxidative cleavage on exposure to light, and riboflavin markedly intensifies this action of light. As little as 50 μg. of riboflavin per 100 cc. of solution can accelerate the destruction of 10 mg. of folic acid. The reaction is much more rapid at *p*H 4.0 than at *p*H 6.5, and is retarded when the air is replaced by nitrogen. The products of the riboflavin-induced photo-oxidation of folic acid are *p*-aminobenzoylglutamic acid and a carbonyl compound, presumably 2-amino-4-hydroxy-6-pteridine-carboxaldehyde. Most of the riboflavin remains undecomposed. A possible mechanism is proposed for this reaction, and other data on folic acid stability are presented.

ATTENTION has lately been focused on the stability problem arising when folic acid is combined with other vitamins in liquid mixtures. Perhaps the earliest published recognition of this problem occurs in the *Calco Technical Bulletin* on folic acid (1) which states that "certain factors of the B complex and also vitamin C have a deleterious effect on folic acid." The vitamin having the most injurious effect was riboflavin, followed, in descending order, by thiamine, ascorbic acid, nicotinamide, pantothenic acid, and pyridoxine. Later, during a study of folic acid stability in combination with other drugs in liquid prescriptions, Scheindlin (2) found that two polyvitamin mixtures exerted a destructive effect on folic acid after three months' storage at room temperature.

The next reference to folic acid instability came, curiously enough, from the field of bacteriology. Koft and Sevag (3), while studying the role of folic acid and *p*-aminobenzoic acid in the inhibition of *Lactobacillus arabinosus* by sulfonamides, noted a spontaneous loss of folic acid in a sterile, neutral culture medium containing, among a variety of ingredients, six of the B complex vitamins, including thiamin and riboflavin. The breakdown of folic acid manifested itself in a liberation of diazotizable amine. Further data on this decomposition were presented in a later paper by these authors (4), but the factors responsible were not determined. It seems reasonable that the vitamins of the culture medium were among the offenders, if not the sole offenders. Thus it appears that interactions between vitamins constitute a problem not only for the pharmacist and the manufacturer of pharmaceuticals, but also for many scientists, such as bacteriologists and biochemists, who have not yet realized that the problem exists.

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Biamonte and Schneller (5) conducted an extensive study of the stability of folic acid in solutions containing individually and conjointly the following B complex vitamins: thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine, and pantothenyl alcohol. Their mixtures were stored in the dark at room temperature and also at 45°. These workers found that thiamine and riboflavin caused considerable decomposition of *dissolved* folic acid. In those acid media where most of the folic acid remained undissolved (*p*H 3.0 and 4.0), it was quite stable. Nicotinamide, pyridoxine, and pantothenyl alcohol were found quite compatible with folic acid. The decomposition of folic acid by thiamine and riboflavin involved cleavage at the methylene linkage, liberating an aromatic amine, presumably *p*-aminobenzoylglutamic acid.

At our laboratories the study of folic acid interaction with other individual vitamins was undertaken, not to gather data on stability, but with the aim of learning what chemical reactions take place between the vitamins. The first pair to be studied was folic acid-ascorbic acid, and it was found (6) that ascorbic acid first cleaves folic acid, producing a diazotizable amine. The amine is subsequently destroyed by further reaction with ascorbic acid or its oxidation products.

The reactions occurring between folic acid and riboflavin are the subject of this report.

EXPERIMENTAL

The Basic Vitamin Mixture.—In order to limit the number of variables, it was considered desirable to employ a reaction mixture of constant composition in most of the experiments. Such a mixture should possess the following characteristics:

1. It must keep the folic acid entirely in solution, since it has been suggested (5) that undissolved folic acid is not readily attacked by riboflavin.
2. It should contain folic acid and riboflavin in equimolar ratio, rather than in any arbitrary numerical ratio.
3. Its *p*H should be in the range of most B complex syrups and elixirs. A *p*H of 4.0 was selected. Riboflavin is quite stable at this *p*H.

TABLE I.—STABILITY OF FOLIC ACID WITH RIBOFLAVIN AT 42°

Sample No.	Time, Days	Free PABG Calculated as Folic Acid, Mg./10 Cc.	Folid Acid Recovered, Mg./10 Cc.	Decomposition, %
Control	0	0.04	1.00	3.8
	14	0.05	1.00	4.7
	28	0.05	1.04	4.6
RB4	0	0.14	0.91	13.3
	14	0.38	0.66	36.5
	28	0.54	0.53	50.5
RE1	0	0.16	0.92	14.8
	14	0.30	0.78	27.8
	28	0.40	0.68	37.0
RI1	0	0.15	0.92	14.0
	14	0.37	0.70	34.5

To meet these requirements the following formula referred to hereafter as "Basic Vitamin Mixture," was developed:

Folic acid[1]	10 mg.
Riboflavin[1]	8.5 mg.
Propylene glycol	50 cc.
Clark-Lubs buffer, *p*H 3.4, q. s.	100 cc.

Folic acid remained in solution in this vehicle at the usual laboratory temperatures. During the winter nights, when room temperature fell below normal, a small amount of folic acid crystallized out, but this was found to have no effect on the results obtained.

Liter quantities of this solution were prepared by dissolving the riboflavin in some of the buffer over a water bath, and adding the propylene glycol. The folic acid, dissolved in a little water with the aid of 10% sodium hydroxide, was then added, and finally the solution was made up to volume with buffer. The final *p*H varied from 3.9 to 4.1.

When small quantities such as 100 cc. were prepared, stock solutions of the vitamins were employed.

In order to minimize metallic ion contamination, all distilled water used for preparing vitamin mixtures was redistilled in all-Pyrex apparatus. All glassware in which vitamin mixtures were prepared and stored was rinsed thoroughly with redistilled water.

Analytical Procedure.—The chemical assay of folic acid is based on the fact that, when treated with zinc and hydrochloric acid, folic acid undergoes cleavage, yielding *p*-aminobenzoylglutamic acid (PABG). The aromatic amino group of PABG is diazotized and then coupled with Marshall's reagent. The red color so produced is measured colorimetrically or spectrophotometrically. Any PABG which may be present *before* reduction of the folic acid is determined concurrently and subtracted from the total PABG to find the amount of PABG which represents undecomposed folic acid. A small percentage of PABG is usually present as an impurity in folic acid. It has been shown also that PABG is formed when folic acid is acted upon by ascorbic acid (6) and by thiamine and riboflavin (5). Thus the chemical assay of folic acid provides a simultaneous assay method for the chief decomposition product of folic acid encountered in mixtures with other vitamins.

The method employed in these studies was based on the original method of Hutchings, *et al.* (7), with the adaptations previously described by two of the present authors (6). In tabulating results (Table I), the values for free PABG present before reduction have been presented under the heading "Free PABG Calculated as Folic Acid." The figures in this column thus represent the amount of folic acid which has undergone decomposition. The numerical sum of this column and the column headed "Folic Acid Recovered" equals total folic acid (both decomposed and undecomposed). The per cent decomposition was calculated as follows:

$$\text{Per cent decomposition} = \frac{\text{free PABG calculated as folic acid}}{\text{total folic acid}} \times 100$$

Folic Acid and Riboflavin at 42°.—At the outset of this investigation, samples of Basic Vitamin Mixture were stored at 42° ± 2°, and assayed at two-week intervals. At the same time, controls having the same formula but no riboflavin were prepared and treated in the same way. One month's storage of a vitamin product at this temperature is widely considered to be equivalent to a year's storage at room temperature (1). Typical data thus obtained are given in Table I.

Two interesting points are brought out by these results:

1. Decomposition of folic acid did occur at *p*H 4.0, since the folic acid was in solution. Biamonte and Schneller (5) found no decomposition at this *p*H level when folic acid was present in solid, undissolved form.

2. The sum of the free PABG calculated as folic acid and of undecomposed folic acid remained constant, within the limits of experimental error. This was also observed by Biamonte and Schneller (5) for folic acid-riboflavin mixtures. On the other hand, when ascorbic acid reacts with folic acid, the PABG which is formed undergoes further degradation (6).

Effect of Light on Basic Vitamin Mixture.—Early in the course of these experiments it was noted that solutions left on the desk top for any length of time before assaying showed a surprisingly high content of free PABG. For example, one solution which was prepared but not assayed until next day yielded 22.7% free PABG. Another solution, allowed to stand over a week-end, gave 40.0% free PABG. Such high decomposition figures at room temperature were surprising in view of the fact that two weeks at 42° usually produced less than 40% decomposition.

Light was the only factor which could be responsible for this anomalous behavior, though at first glance this did not seem very likely. All samples of vitamin mixture had been stored in 4-oz. amber bottles. Furthermore, the laboratory table was not exposed to direct sunlight, and was usually illuminated by electric light. Experiments were therefore designed to determine the possible effect of light on the Basic Vitamin Mixture. Results are presented in Table II.

All vitamin solutions throughout this investigation were stored in amber bottles of the same type

[1] These quantities were permitted to vary by a few tenths of a milligram. The exact amount of folic acid present was determined by chemical assay. Riboflavin was weighed on the analytical balance.

TABLE II.—EFFECT OF LIGHT ON FOLIC ACID-RIBOFLAVIN MIXTURES

Sample No.	Storage Conditions	Time, Days	Free PABG Calculated as Folic Acid, Mg./10 Cc.	Folic Acid Recovered, Mg./10 Cc.	Decomposition, %
RQ (folic acid only)	In light, RT	0	0.03	1.08	2.7
		6	0.05	1.04	4.6
		21	0.06	1.10	5.2
		62	0.18	0.92	16.4
RK1	In light, RT	0	0.04	1.08	3.5
		1	0.08	1.06	7.0
		3	0.17	0.96	15.0
		6	0.59	0.53	52.7
		17	0.92	0.17	84.4
RK2	In dark, 42°	0	0.04	1.08	3.5
		1	0.07	1.07	6.1
		3	0.10	1.06	8.6
		6	0.14	0.99	12.4
		8	0.18	0.95	15.9
		10	0.27	0.84	24.3
		14	0.38	0.75	33.6
		22	0.36	0.75	32.4
RK25[a]	In light, RT	0	0.18	0.95	15.9
		2	0.45	0.69	39.5
		6	0.59	0.54	52.2
		14	0.98	0.15	86.7
RL1	In light, RT	0	0.06	1.00	5.7
		1	0.12	0.92	11.5
		2	0.28	0.77	26.7
		4	0.74	0.31	70.5
		7	0.98	0.06	94.2
RL2	In dark, 42°	0	0.06	1.00	5.7
		1	0.06	0.98	5.8
		2	0.16	0.88	15.4
		4	0.14	0.91	13.3
		7	0.36	0.69	34.3
		9	0.17	0.88	16.2
		14	0.34	0.71	32.4[b]
RL25[c]	In light, RT	0	0.36	0.69	34.3
		7	1.04	0.00	100.0
RO3	In light, RT	0	0.06	0.91	6.2
		5	0.87	0.17	83.6
		11	1.02	0.00	100.0
RO4	In dark, RT	0	0.06	0.91	6.2
		5	0.25	0.80	23.8
		11	0.25	0.81	23.6

[a] Consists of a sample of RK2, withdrawn on the eighth day and stored in the light at room temperature. Thus, decomposition in RK2 after twenty-two days may be compared with that in RK25 after fourteen days.
[b] The fluctuations in RL2 were attributed to the fact that some of the assay samples were drawn from full bottles and others from partially empty bottles which allowed more contact with air.
[c] Consists of a sample of RL2, withdrawn on the eighth day and stored in the light at room temperature.

and were never exposed to any more light than the normal illumination of the laboratory.

Fluorometric assays for riboflavin were not carried out because the fluorescent pteridines formed by cleavage of folic acid would have interfered with such determinations. Instead, the initial riboflavin content of the reaction mixtures was obtained by accurate weighing of dried riboflavin. At the end of the experimental period, samples were analyzed for riboflavin microbiologically[1] (Table III).

Results obtained by two such diverse methods must be compared with the utmost caution, but the indications are that little, if any, riboflavin is destroyed during the accelerated photolysis of folic acid.

Effect of Light on Folic Acid-Riboflavin Mixtures in All-Aqueous Acid Medium.—The following mixtures, ten times as concentrated as Basic Vitamin Mixture, were prepared:

RR1	Folic acid.....	100 mg.	
	NaOH solution, *q. s.*, to dissolve		
	Buffer, *p*H 4.0, *q. s.*	100 cc.	Final *p*H 4.5
RR2 and 3	Folic acid.....	100 mg.	
	NaOH solution, *q. s.*, to dissolve		
	Riboflavin.....	75 mg.	
	Buffer, *p*H 4.0, *q. s.*	100 cc.	Final *p*H 4.8

The buffer was prepared with 0.075% methylparaben as a preservative. A large portion of both vitamins was not in solution in these mixtures. RR1 and RR2 were exposed to light in amber bottles, while RR3 was stored in the dark. All were at room temperature. Results are given in Table IV.

In this series the combined effect of light and riboflavin on folic acid is clearly illustrated. However, since most of the folic acid was undissolved, the reaction proceeded at a comparatively slow rate.

Filtered samples of RR2 produced a reddish-yellow precipitate with 2,4-dinitrophenylhydrazine,

[1] The authors are indebted to Mr. M. E. Avery of Lederle Laboratories for all microbiological assays reported herewith.

indicating the formation of a carbonyl compound upon cleavage of folic acid. RR1 and RR3 produced only traces of this precipitate on standing. Riboflavin, alone, and fresh solutions of folic acid gave no precipitate with the reagent.

TABLE III

Sample	Riboflavin Content in Mg. per 100 Cc. Initial Weight	Final Assay
RK1	8.43	7.5
RK2	8.43	7.9
RL1	8.26	6.2
RL2	8.26	6.5
RO3	9.38	7.2
RO4	9.38	8.3

Another all-aqueous series of mixtures was prepared, having the same quantities of vitamins as the Basic Vitamin Mixture. The final *p*H of these mixtures was 3.8; the riboflavin was entirely dissolved, but some of the folic acid was precipitated. In the sample exposed to light this precipitate redissolved in a few days; in seven days, 100% cleavage of folic acid had occurred. The rapidity of the reaction may be attributed in part to the summer heat, which produced solution temperatures up to 32°.

This solution, after cleavage of the folic acid had been completed, gave a reddish-yellow precipitate with 2,4-dinitrophenylhydrazine.

The Reaction in Aqueous Medium at *p*H 6.5.—The basic vitamin solution at this *p*H level had the following formula:

Folic acid	10 mg.
Riboflavin	8.5 mg.
Clark-Lubs buffer, *p*H 6.4, *q. s.*	100 cc.

Sodium hydroxide solution was used to dissolve the folic acid. The final *p*H of all solutions in this series was 6.4. Methylparaben, 0.075%, was added to the buffer as a preservative.

Controls containing no riboflavin were also prepared. The results of these experiments are presented in Table V.

At the end of the experimental period, samples of LB2 and RT2 gave a reddish-yellow precipitate with 2,4-dinitrophenylhydrazine reagent, indicating the formation of a carbonyl compound.

Effect of Varying the Concentration of Riboflavin.—In this series the Basic Vitamin Mixture of *p*H 4.0 was employed, but the concentration of riboflavin was varied. The experimental details and results are presented in Table VI.

The Reaction under Nitrogen.—A sample of Basic Vitamin Mixture (*p*H 4.1) was prepared, assayed, and divided into three amber bottles. Two of the bottles were fitted with two-hole rubber stoppers carrying glass tubing, and a stream of nitrogen was bubbled through the solution for sixty-six and one-half hours, at which time it was re-assayed. The third bottle, placed near the other two, served as a control. All the solutions were exposed to light. This experiment was later repeated. Results are given in Table VII.

When the experiment was repeated, nitrogen was bubbled through a bottle containing 50% propylene glycol before being passed into the vitamin solution.

TABLE IV.—EFFECT OF LIGHT ON MIXTURES IN ALL-AQUEOUS ACID MEDIUM

Sample No.	Time, Days	Free PABG Calculated as Folic Acid, Mg./Cc.	Folic Acid Recovered, Mg./Cc.	Decomposition, %
RR1	0	0.04	0.94	4.1
	14	0.05	0.89	5.3
	22	0.08	0.86	8.5
RR2	0	0.09	0.93	8.8
	14	0.15	0.83	15.3
	22	0.29	0.67	30.2
RR3	0	0.09	0.93	8.8
	14	0.07	0.91	7.1
	22	0.05	0.92	5.2

TABLE V.—REACTION IN AQUEOUS MEDIUM AT *p*H 6.5

Sample No.	Storage Conditions	Time, Days	Free PABG Calculated as Folic Acid, Mg./10 Cc.	Folic Acid Recovered, Mg./10 Cc.	Decomposition, %
LB1 (folic acid alone)	In light, RT	0	0.02	1.10	1.8
		3	0.02	1.14	1.7
		6	0.04	1.09	3.5
		12	0.02	1.08	1.8
LB2	In light, RT	0	0.04	1.08	3.6
		3	0.27	0.83	24.5
		6	0.35	0.78	30.9
		12	0.45	0.65	40.9
LB3	In dark, RT	0	0.04	1.08	3.6
		3	0.06	1.04	5.5
		6	0.16	0.96	14.3
		12	0.13	0.99	11.6
RT1 (folic acid alone)	In light, RT	0	0.02	1.05	1.9
		7	0.06	1.05	5.4
		13	0.05	1.04	4.6
RT2	In light, RT	0	0.08	1.05	7.1
		7	0.25	0.84	22.9
		13	0.35	0.78	31.0
RT3	In dark, RT	0	0.08	1.05	7.1
		7	0.12	1.02	10.5
		13	0.19	0.89	17.6

TABLE VI.—EFFECT OF VARYING RIBOFLAVIN CONCENTRATION AT *p*H 4.0

Sample No.	Riboflavin, Mg./100 Cc.	Storage Conditions	Time, Days	Free PABG Calculated as Folic Acid, Mg./10 Cc.	Folic Acid Recovered, Mg./10 Cc.	Decomposition, %
LF3	0	In light, RT	0	0.03	1.00	2.9
			3	0.03	1.01	2.9
			14	0.03	0.97	3.0
			37	0.05	0.94	5.1
LF4	0	In dark, RT	0	0.03	1.00	2.9
			3	0.03	1.00	2.9
			14	0.03	0.97	3.0
			37	0.03	0.95	3.1
LF1	0.05	In light, RT	0	0.04	0.99	3.9
			3	0.10	0.91	9.9
			14	0.19	0.82	18.8
			37	0.76	0.23	76.8
LF2	0.05	In dark, RT	0	0.04	0.99	3.9
			3	0.04	0.98	3.9
			14	0.05	0.97	4.9
			37	0.03	0.96	3.0
LG1	0.10	In light, RT	0	0.03	0.97	3.0
			3	0.11	0.93	10.6
			7	0.21	0.80	20.8
			14	0.39	0.63	38.2
			36	0.97	0.03	97.0
LG2	0.10	In dark, RT	0	0.03	0.97	3.0
			3	0.05	0.97	4.9
			7	0.05	0.96	5.0
			14	0.06	0.97	5.8
			36	0.06	0.94	6.0
LG3	0.20	In light, RT	0	0.03	0.99	2.9
			3	0.36	0.68	34.6
			7	0.93	0.06	93.9
			14	1.01	0.04	96.2
LC1	0.20	In light, RT	0	0.02	1.04	1.9
			3	0.10	0.95	9.5
			7	0.13	0.90	12.6
			10	0.20	0.80	20.0
LG4	0.20	In dark, RT	0	0.03	0.99	2.9
			3	0.06	0.97	5.8
			7	0.03	0.97	3.0
			14	0.06	0.96	5.9
			36	0.06	0.96	5.9
LC2	2.0	In light, RT	0	0.04	0.97	4.0
			3	0.27	0.78	25.7
			7	0.75	0.29	72.1
			10	1.02	0.02	98.1
LE4	8.5	In light, RT	0	0.06	0.99	5.7
			3	0.60	0.45	57.1
			6	1.05	0.02	98.1
LC4	8.5	In dark, RT	0	0.08	0.97	7.6
			3	0.06	1.00	5.7
			7	0.09	0.96	8.6

These results show that oxygen is one of the factors contributing to the speed of the photolytic reaction between folic acid and riboflavin, although the reaction occurs even when oxygen is excluded.

Effect of *p*-Aminobenzoic Acid on Photo-oxidation of Folic Acid.—Quite recently Rauen and Kimbel (8) have reported on the protective action of PABA against photo-oxidation of folic acid. They followed the reaction by measuring the fluorescence produced by the pteridines which result from photo-oxidative cleavage of folic acid. According to these authors, solutions containing 15 mg. sodium folate and 20 mg. sodium *p*-aminobenzoate per 2 cc. showed only negligible increase in fluorescence during forty-one days' storage in direct daylight in a window.

At our laboratories this experiment was repeated, but decomposition was measured by the colorimetric assay. The results obtained by Rauen and Kimbel were confirmed, since after fourteen days the amount of decomposition was only 2.6%. A control containing no PABA lost 58.4% of its folic acid in the same period of time.

The *p*H at which these results were obtained was 8.8. It will be of interest to learn whether PABA exerts any protective action in acid medium and in the presence of riboflavin.

DISCUSSION

While light and riboflavin, individually, each exert a cleaving effect on folic acid, it has been shown by the foregoing experiments that the combined effect of both these factors on folic acid is not additive, but synergistic. Before considering the riboflavin-folic acid reaction in detail, the effect of

TABLE VII.—THE REACTION UNDER NITROGEN

Sample	Storage Conditions	Time	Free PABG Calculated as Folic Acid, Mg./10 Cc.	Folic Acid Recovered, Mg./10 Cc.	Decomposition, %
Original solution		0 hr.	0.05	1.09	4.4
Control	Under air	66.5 hr.	1.10	0.02	98.2
1	Under N_2	66.5 hr.	0.85	0.80[a]	51.5
2	Under N_2	66.5 hr.	0.59	0.52	53.2
		Repetition of Experiment			
Original solution		0 day	0.09	0.91	9.0
Control	Under air	3 days	1.01	0.04	96.2
1	Under N_2	3 days	0.38	0.67	36.2

[a] Average of two assays. The high values for PABG and folic acid are due to concentration of the solution by evaporation as the current of nitrogen was passed directly into this bottle. From this bottle, the gas (now saturated with vapor) was passed into sample 2, which gave normal assay values.

light on each of these vitamins individually must be discussed.

Stokstad, *et al.* (9), were the first to report that folic acid solutions were rapidly decomposed with the splitting off of PABG when exposed to daylight. Lowry, Bessey, and Crawford (10) made a thorough study of this reaction under ultraviolet light, identifying the pteridine reaction products chiefly by their fluorescence spectra, absorption spectra, and behavior toward the enzyme xanthine oxidase. These workers reported that the reaction proceeds most rapidly at *p*H 3, but can be carried to completion at both *p*H 1 and *p*H 7. The reaction was formulated as follows:

Folic acid
↓ U. V. light
2-Amino-4-hydroxy-6-pteridinecarboxaldehyde + diazotizable amine
↓ U. V. light
2-Amino-4-hydroxy-6-pteridinecarboxylic acid
↓ U. V. light
2-Amino-4-hydroxypteridine

The oxidative photolysis proceeds very slowly in the complete absence of dissolved oxygen. The slow reaction which does occur was attributed by the authors to the presumable photolytic release of oxygen from the water.

Riboflavin is very sensitive to light and undergoes irreversible decomposition on irradiation with ultraviolet rays or visible light. Irradiation of alkaline solutions splits off the D-ribityl side chain, forming lumiflavin. Irradiation of neutral or acid solutions of riboflavin produces lumichrome (6,7-dimethyl alloxazine) (11). Both lumiflavin and lumichrome are devoid of biological activity.

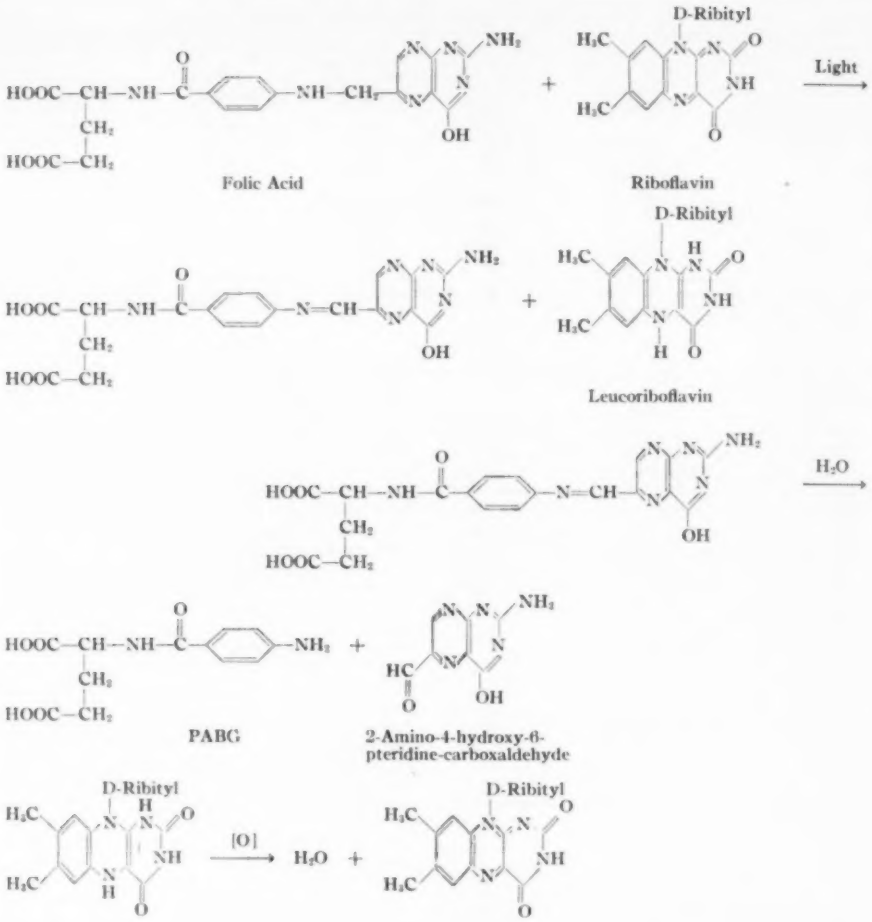
About fifteen years ago it was discovered that riboflavin has the ability to catalyze the photo-oxidation of ascorbic acid, and that this fact is responsible for the rapid loss of ascorbic acid which occurs in milk. Hopkins (12) found observable acceleration of vitamin C oxidation in diffused daylight when as little as 70 μg. of riboflavin per 100 cc. of solution was present. He suggested that the mechanism by which riboflavin promotes the reaction involves the activation of oxygen. Hand, Guthrie, and Sharp (13) studied the effect of riboflavin on the photochemical oxidation of ascorbic acid, using pure compounds and artificial light. These workers determined that air, light, and riboflavin are all required for this oxidation to take place. Since neither ascorbic acid nor oxygen can absorb any of the visible light which filters through heavy glass bottles, this light has no effect on ascorbic acid. Riboflavin, however, absorbs blue light, and thus, according to Hand, *et al.*, promotes the photo-oxidation of ascorbic acid.

This action of riboflavin is analogous to its action on folic acid, as it has unfolded itself during the studies herein reported. When stored in amber bottles, folic acid solutions were not affected by the diffuse daylight and electric light of the laboratory. When riboflavin was added, however, folic acid became very unstable in these same bottles and under the same lighting conditions. Ultraviolet light could not be responsible, as it was filtered out first by the glass of the laboratory windows and then by the amber glass, which, in a thickness of 2 mm., transmits no more than 5% of all wave lengths below 450 mμ (14). This glass does transmit much of the visible light above 500 mμ, and some of this light, absorbed by riboflavin, appears responsible for the cleavage of folic acid.

When the proportion of riboflavin to folic acid was equimolar (8.5 mg. : 10 mg.) the most rapid photolysis was obtained. Yet even after cleavage of folic acid was complete, the riboflavin activity could be recovered (by microbiological assay) essentially unreduced. Furthermore, definite acceleration of folic acid photo-oxidation occurred with as little as 50 μg. riboflavin to 10 mg. folic acid, or a molar ratio of 1 to 166. Even in this case, cleavage of folic acid, though slower, proceeded to completion. These facts have prompted the conclusion that the role of riboflavin is that of a hydrogen-acceptor, as it is in many biochemical reactions. The reduction of riboflavin to its dihydro or leuco form is reversible and would lead to no loss of microbiological activity.

The fact that the reaction is slowed but not stopped by exclusion of air may indicate that air is not the source of oxygen for the primary oxidation, but rather supplies oxygen for the regeneration of riboflavin from leucoriboflavin. Reversible reduction to leucoriboflavin could account for the ability of 50 μg. of riboflavin to promote the oxidation of 10,000 μg. of folic acid.

The following equations, which fit the observed experimental results, represent a possible mechanism



for the riboflavin-catalyzed photo-oxidation of folic acid.

CONCLUSIONS

1. Under the combined effect of light and riboflavin, folic acid undergoes rapid oxidative cleavage, with the formation of *p*-aminobenzoylglutamic acid and a carbonyl compound, presumably 2-amino-4-hydroxy-6-pteridinecarboxaldehyde.

2. This reaction occurs much more rapidly at *p*H 4.0 than at *p*H 6.5.

3. The reaction is retarded, but not halted, by exclusion of air.

4. As little as 50 μg. of riboflavin per 100 cc. of solution can accelerate the complete cleavage of 10 mg. of folic acid, when exposed to a light intensity which in itself has no effect on folic acid.

5. A possible mechanism has been proposed for this reaction, and other data on folic acid stability in the presence of riboflavin have been presented.

6. The report that *p*-aminobenzoic acid can protect folic acid from the action of light in alkaline medium has been confirmed.

REFERENCES

(1) Foss, N. E., Schneller, G. H., and Sullivan, C. F., "Folic Acid," *Calco Technical Bulletin* No. 721, Nov. 1947 p. 9.
(2) Scheindlin, S., *Am. J. Pharm.*, **120**, 107(1948).
(3) Koft, B., and Sevag, M. G., *J. Am. Chem. Soc.*, **71**, 3243(1949).
(4) Koft, B. W., Sevag, M. G., and Steers, E., *J. Biol. Chem.*, **185**, 9(1950).
(5) Biamonte, A. R., and Schneller, G. H., THIS JOURNAL, **40**, 313 (1951).

(6) Scheindlin, S., and Griffith, I., *Am. J. Pharm.*, **123**, 78(1951).
(7) Hutchings, B. L., *et al.*, *J. Biol. Chem.*, **168**, 705 (1947).
(8) Rauen, H. M., and Kimbel, K. H., *Z. Physiol. Chem.*, **286**, 215(1951).
(9) Stokstad, E., *et al.*, *J. Biol. Chem.*, **167**, 877(1947).
(10) Lowry, O. H., Bessey, O. A., and Crawford, E. J., *ibid.*, **180**, 389 (1949).
(11) "The Vitamins," American Medical Association, Chicago, 1939, p. 263.
(12) Hopkins, F. G., *J. Soc. Chem. Ind.*, **56**, 934(1937).
(13) Hand, D. B., Guthrie, E. S., and Sharp, P. F., *Science*, **87**, 439(1938).
(14) "Light Transmission of Commercial Container Glasses," brochure prepared by Armstrong Cork Company.

Studies on Single Oral Doses of a High Gel Point Methylcellulose*

By H. F. KNIGHT, Jr.,† H. C. HODGE,† E. P. SAMSEL,‡ R. E. DeLAP,‡ and D. D. McCOLLISTER‡

A high gel point methylcellulose (Methocel HG) was given in single doses to normal healthy young adults. Only minor symptoms were reported, e. g., mild laxative effect, mild constipating effect, and a few instances of commonplace symptoms such as cramping, flatus, headache, and tenesmus which apparently were not related to the ingestion of Methocel HG. Essentially all of the ingested Methocel HG was eliminated in the feces within ninety-six hours following administration.

METHOCEL HG[1] is a water-soluble cellulose ether, recently available, that has found uses in the food and pharmaceutical industries. The physiological inertness of the compound has already been demonstrated in chronic toxicity studies using rats (two years) and dogs (one year) (2). A quantitative demonstration of the stability in the gastrointestinal tract has been made in the current study by measuring the recovery of Methocel HG in the feces following the ingestion of large doses by human subjects. It is instructive to compare these results with the animal (3, 4) and human studies (5) of Methocel which have shown that it passes through the human digestive tract virtually unchanged, i. e., practically all of the methoxyl groups of the ingested methocellulose were recovered from the feces.

PROCEDURE AND METHODS

The present study was carried out on human subjects ingesting single doses of Methocel HG to determine the recovery in feces. Twenty-five adult students, 23 male and 2 female, were screened regarding general health, especially as to gastrointestinal tract normality. Each individual was given three graduated doses of Methocel HG[2] ranging from 0.6 to 8.9 Gm. The time interval between doses was at least one week. Each subject was instructed to avoid an unusual intake of cellulose-containing food, food having a laxative effect, or excessive fluids during the period of the experiment. For each dose, the subject kept a record of his bowel habits, any side effects, and his impressions of the material's activity.

Following each dose of Methocel HG, stool specimens were collected at approximately twenty-four-hour intervals for seventy-two or ninety-six hours. In the first tests no pre-dose specimen was collected; in the latter tests a twenty-four-hour control stool specimen was collected before each dose. The examination of specimens and interpretation of other data for evaluating effects upon the gastrointestinal tract were made by one individual (H. F. K., Jr.). The following criteria were used to judge each dose of Methocel HG: (*a*) net weight of stool specimens; (*b*) consistency and moisture content of stool specimens; (*c*) variations from usual number of daily bowel movements; (*d*) subjective opinion as to the size and consistency of stool; (*e*) subjective opinion as to effect of the material.

The collected samples were dried at 95–105° to constant weight (forty-eight to sixty hours). In determining the quantitative amounts of Methocel HG present in the samples of dried feces, particular attention was given to the larger dosage levels. The analytical procedure consisted of a methoxyl determination made directly on the feces samples. The dried sample was broken up, transferred to a mortar or a Wiley Micro Mill, and then ground to a powder.

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† Division of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.
‡ Cellulose Products and Biochemical Research Laboratories, the Dow Chemical Company, Midland, Mich.

[1] Methocel is the registered trade mark of the Dow Chemical Company. Methocel HG is a chemically modified Dow methocellulose similar to Methocel (1) in all properties except the gel point, which is 10 to 15° higher than that of comparable solutions of Methocel. This increase improves the compatability with many pharmaceutical ingredients and gives better storage properties, especially in warm climates.

[2] Methocel HG tablets weighing 0.22 Gm. each were supplied by the Dow Chemical Company. These tablets consisted of 98.2% Methocel HG (25 c. p. s. viscosity type) and 1.8% moisture. The methoxyl content of the tablets was 30.4%; the theoretical value for two methoxyl groups/glucose unit is 32.6%.

TABLE I.—METHOCEL HG RECOVERED FROM FECES OF HUMAN SUBJECTS INGESTING LARGE SINGLE DOSES

Subject	Methocel HG, Gm.	Gastrointestinal Effect	Methocel HG Recovered, Gm. 24 Hr.	48 Hr.	72 Hr.	96 Hr.	Total	% Recovery Uncorr.	Corr.[a]
R. H.	3.0	None	0.4	1.4	0.7	nil	2.5	83	89
R. W.	3.5	None	1.9	1.0	nil	nil	2.9	83	89
H. U.	4.1	Mild laxation	0.6	1.7	1.4	0.5	4.2	102	110
R. Z.	5.0	Mild constipation	0.2	2.3	1.7	0.2	4.4	88	95
H. K.	5.0	None	0.2	2.2	2.3	nil	4.7	94	101
H. G.	6.1	Mild constipation	nil	1.5	3.6	nil	5.1	84	90
M. H.	7.2	None	1.5	2.7	2.5	nil	6.7	93	100
M. W.	7.2	Mild laxation	0.3	3.7	2.7	0.1	6.8	94	101
R. S.	8.0	None	2.6	4.8	0.1	nil	7.5	94	101
R. S.	8.9	None	2.4	5.6	0.1	nil	8.1	91	98
R. K.	8.9	Mild constipation	nil	1.6	2.0	4.1	7.7	86	92

[a] Corrected for average recovery (93%) of known amounts added to feces.

After thorough mixing to insure uniformity, duplicate samples of 0.25 to 0.30 Gm. each were placed in small capsules, and methoxyl determinations were made according to the method of Samsel and McHard (6).

The amount of Methocel HG recovered was calculated as follows:

$$\frac{\%\text{ Methoxyl in Feces Sample} - \%\text{ Methoxyl in Control Sample}}{30.4 \times 0.982} \times \text{Total Wt. of Feces Sample}$$

Nine determinations were made in which known amounts (3 to 9 Gm.) of Methocel HG were added to control samples of feces obtained from individuals not ingesting the experimental material. Each sample was divided into approximately two parts, one portion being used as a blank, and a known amount of Methocel HG being added to the other portion. An average recovery of 93% of the added Methocel HG was obtained (standard error ±2%) with a range of 83 to 104%.

RESULTS

Gastrointestinal Effects.—A total of 76 doses of Methocel HG was ingested by the 25 young adults. Following 49 of the doses, no effect whatsoever was noted. A laxative effect was produced in 11 cases and constipation in 16 cases; these effects were described as mild in degree. In no case was there a marked diarrhea or prolonged constipation. Those subjects having transient constipation usually had a large, soft stool seventy-two to one hundred and twenty hours post-ingestion, with a subsequent return to normal bowel habits.

Side Effects.—On a few occasions, subjects reported side effects such as cramping, flatus, pruritus ani, headache, tenesmus, or urgency. Because of the low incidence and the minor nature of these commonplace symptoms, and because the reported instances were in no way related to the size of the dose or to the slight laxative or constipating effects, these symptoms cannot be attributed to the ingestion of Methocel HG.

Recovery from Feces.—The results of the analyses for Methocel HG in feces samples from the higher dosage levels (3.0 to 8.9 Gm.) are given in Table I; all of the analytical data collected are included. The average total recovery (uncorrected) of methoxyl groups was equivalent to 90% of the ingested dose, a value which was not significantly different statistically from the 93% mean recovery of known amounts added to feces ($P = 0.29$, as determined by the Fisher t-test). Thus, the average total recovery (corrected) of methoxyl groups in the feces was equivalent to 97% of the ingested dose with a range of 89 to 110%.

DISCUSSION

Most important is the fact that essentially all of the Methocel HG ingested by the human subjects was recovered in the feces within ninety-six hours post-ingestion. This finding is in excellent agreement with those of Machle, *et al.* (5), who described accumulated recoveries of 70 to 90% within the first two or three days following the ingestion of 10 Gm. of methylcellulose. They analyzed the feces over a period of two to four weeks following a single dose, but found no methylcellulose after the fourth day. Only 4 of the 11 subjects in the current study had detectable amounts of Methocel HG in the feces on the fourth day; moreover, the over-all recoveries were almost quantitative. In view of the clinical use of methylcellulose as a bulk laxative, the delay time in passage should be noted; frequent instances were found in which an appreciable fraction of the dose of Methocel HG was excreted between the forty-eighth and the seventy-second hour.

SUMMARY

Twenty-five normal, healthy young adults were given single doses of Methocel HG ranging from 0.6 to 8.9 Gm. The results from the recovery study show that Methocel HG is quite similar to Methocel in physiological properties. The data suggest that it is not absorbed to any significant extent from the human digestive tract; essentially all of the ingested Methocel HG was found by quantitative analyses to have been eliminated in the feces within ninety-six hours following single doses of 3.0 to 8.9 Gm.

Mild laxative or mild constipating effects were noted in several cases; minor side effects

were reported occasionally but these apparently were not related to the ingestion of Methocel HG.

REFERENCES

(1) The Dow Chemical Company, Midland, Mich., "The New Methocel," a review, 1949.
(2) Hodge, H. C., Maynard, E. A., Wilt, W. F., Jr., Blanchet, H. J., Jr., and Hyatt, R. E., *J. Pharmacol. Exptl. Therap.*, **99**, 112(1950).
(3) Deichmann, W., and Witherup, S., *J. Lab. Clin. Med.*, **28**, 1725(1943).
(4) Bauer, R. O., and Lehman, A. J., THIS JOURNAL, **40**, 257(1951).
(5) Machle, W., Heyroth, F. F., and Witherup, S., *J. Biol. Chem.*, **153**, 551(1944).
(6) Samsel, E. P., and McHard, J. S., *J. Ind. Eng. Chem., Anal. Ed.*, **14**, 750(1942).

Experiments with Khellin. III. The Formation of Desmethylisokhellin from Khellin*

By HAMED ABU-SHADY† and TAITO O. SOINE‡, §

The formation of desmethylisokhellin by the action of 48 per cent hydrobromic acid on khellin is reported. Experimental evidence is presented to show that desmethylisokhellin is 5-hydroxy-6-methoxy-2-methylfuro[2′:3′:7:8] chromone. A route is suggested for the transformation of khellin to the diphenol corresponding to isokhellin.

KHELLIN (I) is readily converted to a monophenol, desmethylkhellin (II), when refluxed with about 40 per cent hydrobromic acid (sp. gr., 1.38). Evidence to show that desmethylkhellin is 5-hydroxy-8-methoxy-2-methylfuro-[3′:2′:6:7]chromone was adduced in earlier communications (1, 2).

It has now been determined that khellin, when refluxed with 48 per cent hydrobromic acid (sp. gr., 1.49) under similar conditions, yields a second monophenol as the major product. This new monophenol was found to be isomeric with desmethylkhellin and was tentatively named desmethylisokhellin because it yielded isokhellin (5,6-dimethoxy-2-methylfuro[2′:3′:7:8]chromone) when methylated by the methyl iodide-potassium carbonate method. The structure of isokhellin has been established previously (2). On the other hand, no appreciable reaction was noted when desmethylisokhellin was treated with diazomethane. The failure of this phenol to react with diazomethane, coupled with its slight solubility in aqueous sodium hydroxide solution, was considered to be suggestive evidence of a hydrogen bond between the phenolic hydroxyl group and an adjacent carbonyl group. Evidence supporting this view was found in the infrared spectrogram of the phenol. No absorption was noted in the region for free hydroxyl groups, but a feeble absorption in the region of 3,100 cm.$^{-1}$ was accredited to the bonded hydroxyl group.[1]

Because desmethylisokhellin yields isokhellin on methylation and contains only one phenolic hydroxyl group, it may be represented by either of two formulas, III or VI. The former (III) is regarded as being correct because it satisfies the requirement of hydrogen bond formation and because it takes into account the formation of desmethylkhellin (II) as an intermediate. Therefore, desmethylisokhellin is 5-hydroxy-6-methoxy-2-methylfuro[2′:3′:7:8]chromone. A chemical proof of the correctness of this formula is under way.

The formation of desmethylisokhellin in the demethylation procedure described suggests a plausible route for the transformation of khellin to the diphenol (IV) corresponding to isokhellin. This transformation was first noted by Clarke and Robertson (3) when they attempted to obtain the diphenol of khellin by refluxing khellin with hydriodic acid. It is suggested that the changes shown by formulas I through IV outline the changes taking place.

EXPERIMENTAL

The Action of 48% Hydrobromic Acid on Khellin.—Ten grams of khellin was dissolved in 100 cc. of 48% hydrobromic acid (sp. gr., 1.49) and the deep red solution was refluxed gently for ten minutes. The solution was then allowed to stand in the refrigerator overnight. A yellow crystalline residue

* Received March 28, 1952, from the College of Pharmacy, University of Minnesota, Minneapolis.
† Lecturer in Pharmacognosy, Fouad I University, Cairo, Egypt, and Fellow of the American Foundation for Pharmaceutical Education, 1949–1951.
‡ Professor of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota.
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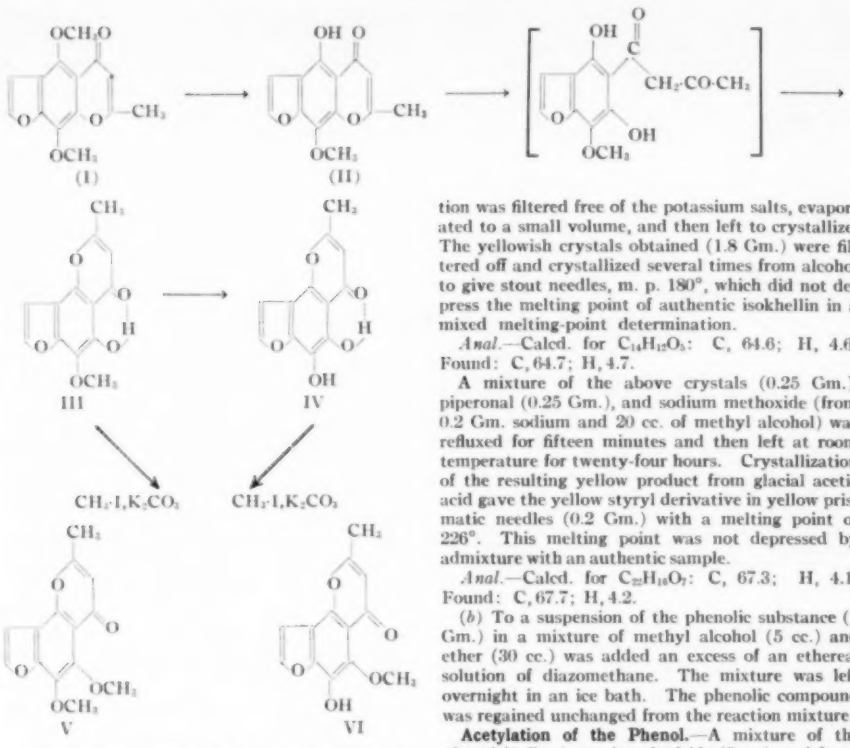
[1] The authors are indebted to Professor B. L. Crawford, Department of Physical Chemistry, University of Minnesota, for the interpretation of the infrared spectrogram.



(1.4 Gm.) was obtained, which, after two crystallizations from glacial acetic acid, gave a melting point of 201°. A mixed melting point determination with desmethylkhellin showed no depression of the melting point, and it was concluded that this product was desmethylkhellin. The acid mother liquor obtained from the crystalline residue was then diluted with a large volume of distilled water and kept in the refrigerator for a few days. The supernatant liquid was then decanted from the yellow resinous material (5.8 Gm.) that had separated. This resinous material hardened on standing and was crystallized twice from glacial acetic acid and twice from acetone to give bright yellow prisms, m. p. 177°. An alcoholic solution of these crystals gave a dark green color with ferric chloride solution. The crystals were only slightly soluble in cold 5% sodium hydroxide solution but were more soluble on warming. They dissolved in concentrated sulfuric acid to give a yellow solution turning green, then blue, and finally reddish brown, on heating. Unlike virtually all other 2-methyl chromones, this compound gave no coloration with sodium hydroxide pellets and a few drops of water. The infrared determination of this substance was run in Nujol mull.

Anal.—Calcd. for $C_{12}H_{10}O_5$: C, 63.4; H, 4.1; OCH_3, 12.6. Found: C, 63.7; H, 4.0; OCH_3, 12.3.

Methylation of the Phenol.—(*a*) A mixture of the phenol (2 Gm.), methyl iodide (5 cc.), anhydrous potassium carbonate (5 Gm.), and acetone (100 cc.) was refluxed for eighteen hours. The acetone solution was filtered free of the potassium salts, evaporated to a small volume, and then left to crystallize. The yellowish crystals obtained (1.8 Gm.) were filtered off and crystallized several times from alcohol to give stout needles, m. p. 180°, which did not depress the melting point of authentic isokhellin in a mixed melting-point determination.

Anal.—Calcd. for $C_{14}H_{12}O_5$: C, 64.6; H, 4.6. Found: C, 64.7; H, 4.7.

A mixture of the above crystals (0.25 Gm.), piperonal (0.25 Gm.), and sodium methoxide (from 0.2 Gm. sodium and 20 cc. of methyl alcohol) was refluxed for fifteen minutes and then left at room temperature for twenty-four hours. Crystallization of the resulting yellow product from glacial acetic acid gave the yellow styryl derivative in yellow prismatic needles (0.2 Gm.) with a melting point of 226°. This melting point was not depressed by admixture with an authentic sample.

Anal.—Calcd. for $C_{22}H_{16}O_7$: C, 67.3; H, 4.1. Found: C, 67.7; H, 4.2.

(*b*) To a suspension of the phenolic substance (1 Gm.) in a mixture of methyl alcohol (5 cc.) and ether (30 cc.) was added an excess of an ethereal solution of diazomethane. The mixture was left overnight in an ice bath. The phenolic compound was regained unchanged from the reaction mixture.

Acetylation of the Phenol.—A mixture of the phenol (1 Gm.), acetic anhydride (5 cc.), and fused sodium acetate (0.2 Gm.) was refluxed for one-half hour. The reaction mixture was then poured into crushed ice to decompose the excess acetic anhydride. The crystalline acetate was separated and crystallized several times from alcohol to yield slightly yellow prisms, m. p. 147°.

Anal.—Calcd. for $C_{13}H_{12}O_6$: C, 62.5; H, 4.2. Found: C, 62.3; H, 4.1.

SUMMARY

1. Desmethylisokhellin, prepared from khellin by the action of 48 per cent hydrobromic acid, has been described and its relationship to isokhellin demonstrated.

2. Evidence has been submitted to show that desmethylisokhellin is 5-hydroxy-6-methoxy-2-methylfuro[2′:3′:7:8]chromone.

3. A plausible series of transformations by which khellin is converted to the diphenol corresponding to isokhellin by the action of hydriodic acid has been suggested.

REFERENCES

(1) Abu-Shady, H., and Soine, T. O., THIS JOURNAL, **41**, 325(1952).
(2) Abu-Shady, H., and Soine, T. O., *ibid.*, **41**,403(1952).
(3) Clarke, J. R., and Robertson, A., *J. Chem. Soc.*, **1949**, p. 302.

The Fruit of *Hyoscyamus muticus* L., Its Macro- and Micromorphology*

By SHAFIK I. BALBAA† and A. HIFNY SABER‡,§

The gross morphology and micromorphology of the fruit and seed of *Hyoscyamus muticus* are described. The differentiation of the fruit from that of *H. niger* is emphasized. The descriptions are illustrated by drawings of significant macroscopic and microscopic characteristics useful for identification and for distinguishing between *H. muticus* and *H. niger*.

THE FRUIT of *Hyoscyamus muticus* L. is of common occurrence in variable proportions in the commercial drug, Egyptian henbane, "Herba Hyoscyami mutici," its presence being permissible as it is equally active and contains the same active constituent.

The published work concerning the macromorphology (gross morphology) of this fruit is fragmentary and the illustrations given are inadequate; in addition, its histology has not been elucidated (1–3). Consequently it was thought that some useful purpose would be served by making a thorough investigation of the fruit with a view to recording those characteristics by which it may best be distinguished both in the whole and in the ground conditions.

MACROMORPHOLOGY OF FRUIT

The fruit is a pyxidium, usually about 3 to 3.5 cm. long, which remains enclosed in, but not adnate with, the persistent pubescent calyx (Fig. 1, *A*), which is more rigid and larger than the flower. The fruit is derived from a superior bicarpellary ovary and is bilocular, containing numerous small seeds attached to an axile placenta. The pyxidium is oblong or oval, more or less cylindrical, about 0.8 to 1.8 cm. long and 0.4 to 0.8 cm. wide, and slightly laterally compressed. It shows two longitudinal shallow grooves on the compressed sides and a minute depression at the apex, from which arises an erect stiff paler apiculus, the remains of the style (Fig. 1, *A*, *B*). At the base of the fruit a thickened ring is usually noticed, which is the remains of the nectary (Fig. 1, *E*).

The fruit dehisces transversely, the upper part separating as a lid (Fig. 1, *B*, *C*, *F*), leaving the lower part of the fruit with most of the seeds enclosed within the calyx and attached to the rachis by a very short stalk or pedicel (Fig. 1, *E*). The line of dehiscence (Fig. 1, *E*) appears as a transverse ring in the upper part of the fruit usually at about one-fourth to one-half the length of the pyxis measured from the apex. The rim of the lid is not even, but shows two projections fitting into two corresponding notches in the lower part of the fruit at the position where the septum is attached to the pericarp. Also, two other shallow notches may be observed between the two projections and into which fit two corresponding projections present on the rim of the lower part of the fruit.

The pericarp is yellowish-green to yellow to light brown, thin and membranous, but thicker and rigid in the lid region. The outer surface is glabrous, usually longitudinally striated, and shows several darker parallel longitudinal lines corresponding to the veins, two of these being more prominent than the others, and corresponding to the midribs of the two carpels. The pericarp also shows reticulations in the lower part, due to the impressions of the numerous seeds which are enclosed within it (Fig. 1, *A*, *E*).

The inner surface of the pericarp is very smooth, shining, and shows numerous indentations in the lower part, due to the seeds.

The outline of the transversely cut fruit is nearly cir cularor more or less oval, showing two shallow, narrow notches on the outer surface (Fig. 1, *G*, *H*). The cavity of the fruit is divided into two locules by a septum which joins the pericarp at the position of the notches, and extends the whole length of the fruit except for a very short distance, about 1 mm., at the apex, where the fruit is unilocular (Fig. 1, *C*, *H*).

The septum is very thin and membranous except in the lid region, where it is thick and rigid near the pericarp; this rigid part remains attached to the lid on dehiscence (Fig. 1, *C*).

The placenta is axile, projecting into the cavity of the fruit on either side of the septum and bearing the seeds (Fig. 1, *D*, *F*). It is soft and fleshy but becomes dry and more or less stiff on ripening and drying, and together with the membranous septum is usually seen projecting beyond the lower part of the pyxis after dehiscence (Fig. 1, *E*).

MICROMORPHOLOGY OF FRUIT

The calyx, which encloses the fruit, resembles that of the flower (4), except that it has developed groups of wood fibers, each fiber with fairly thick lignified walls, a wide lumen, and a length up to 1,320 μ, occasionally up to 2.3 mm. long and 3–24 μ wide; some fibers have forked tips (Fig. 2, *A*, *B*, *F*).

The pedicel, after the withering of the corolla, continues to grow in thickness and becomes harder

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† Lecturer in Pharmacognosy, Faculty of Medicine, Fouad I University, Cairo, Egypt. Present address: graduate student, College of Pharmacy, University of Florida, Gainesville.

‡ Professor of Pharmaceutical Botany, Faculty of Medicine, Fouad I University.

§ The authors wish to express their thanks to Dr. I. R. Fahmy Bey, Head of the Pharmacognosy Department, Faculty of Medicine, Fouad I University, for encouraging this work, and to the Horticulture Section, Ministry of Agriculture, Cairo, Egypt, for supplying some of the material.

and more woody on the development of the fruit (Fig. 2, *C*, *D*). In the pedicel of the fruit, the outer part of the xylem is formed mainly of fibers, together with a few tracheids and occasional vessels. The fibers are mostly forked or branched at the tips (Fig. 2, *F*), and have a wide lumen and distinctly lignified walls.

Pericarp.—The pericarp of the fruit of *Hyoscyamus muticus* L. is 220–340 μ thick in the lower part and may reach up to 531 μ in thickness at the base. In the lid, it is 250 μ thick near the line of dehiscence and up to 920 μ at the apex. It consists of an outer and an inner epidermis enclosing between them the mesocarp, which is formed of numerous layers of parenchymatous cells and is traversed longitudinally by numerous vascular bundles. The number of layers of the mesocarp varies according to the position in the pericarp. There are 6 to 11 layers in the middle and up to 19 layers at the base. In the lid, the mesocarp is formed of two parts; the outer consisting of 10 to 20 layers of parenchyma, sometimes accompanied by collenchyma, and the inner being hard and composed of 1 to 4 layers of lignified sclerenchymatous cells (Fig. 3, *A*, *B*).

Outer Epidermis (Epicarp).—The outer epidermis (Fig. 3, *A*, *B*, *C*, *D*, *E*, *F*, *G*) consists of a single layer of axially elongated polygonal cells, with mostly 4 to 5 sides which are almost straight or slightly curved (Fig. 3, *G*). The cells vary in size according to their position; the biggest cells, in the middle of the fruit, are usually 135–266 μ long, 33–116 μ broad, and 26–43 μ high, but gradually be-

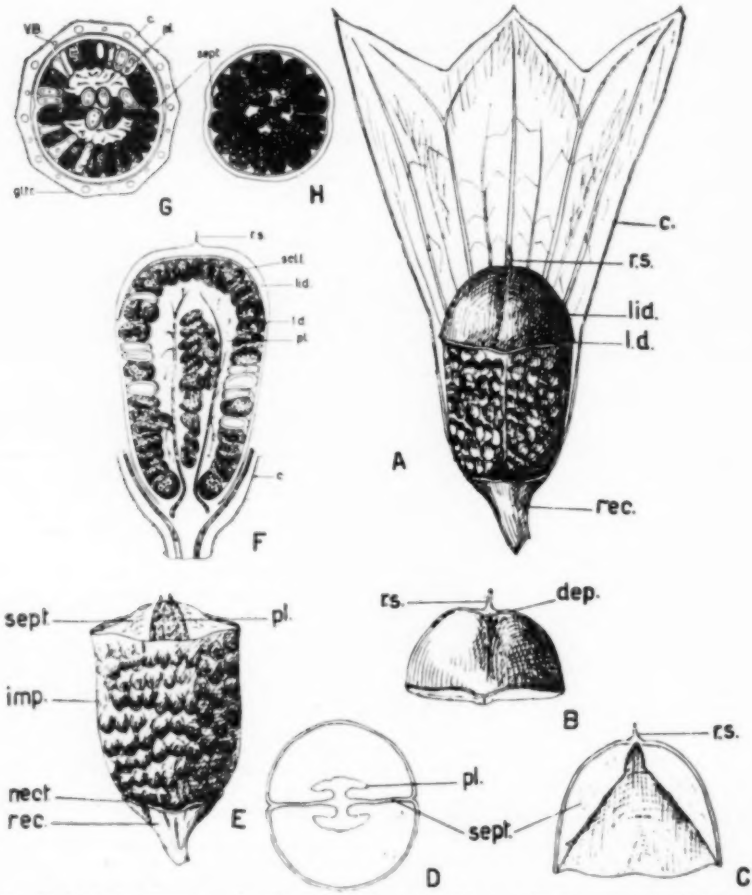

Fig. 1.—Fruit of *Hyoscyamus muticus* L. (*A*) Entire fruit with part of calyx removed; (*B*) lid of pyxis; (*C*) lid in median cut showing septum; (*D*) transverse cut of lower part of fruit; (*E*) lower part of pyxis with its lid removed; (*F*) fruit cut longitudinally; (*G*) lower part of fresh fruit with its calyx cut transversely; (*H*) lid of pyxis separated and still containing seeds; *c.*, calyx; *dep.*, depression; *gl. tr.*, glandular trichomes; *imp.*, impressions of the seeds; *l. d.*, line of dehiscence; *lid*, lid; *nect.*, nectary; *pl.*, placenta; *r. s.*, remains of style; *rec.*, receptacle; *scl. l.*, sclerenchymatous layer; *sept.*, septum; *v. b.*, vascular bundles; all × 3.

come smaller upward as well as toward the base, where they measure 20–100 μ long, 20–60 μ broad, and 33–63 μ high. On either side of the line of dehiscence, the outer epidermal cells abruptly become much smaller and form a zone of a few layers of somewhat transversely elongated cells.

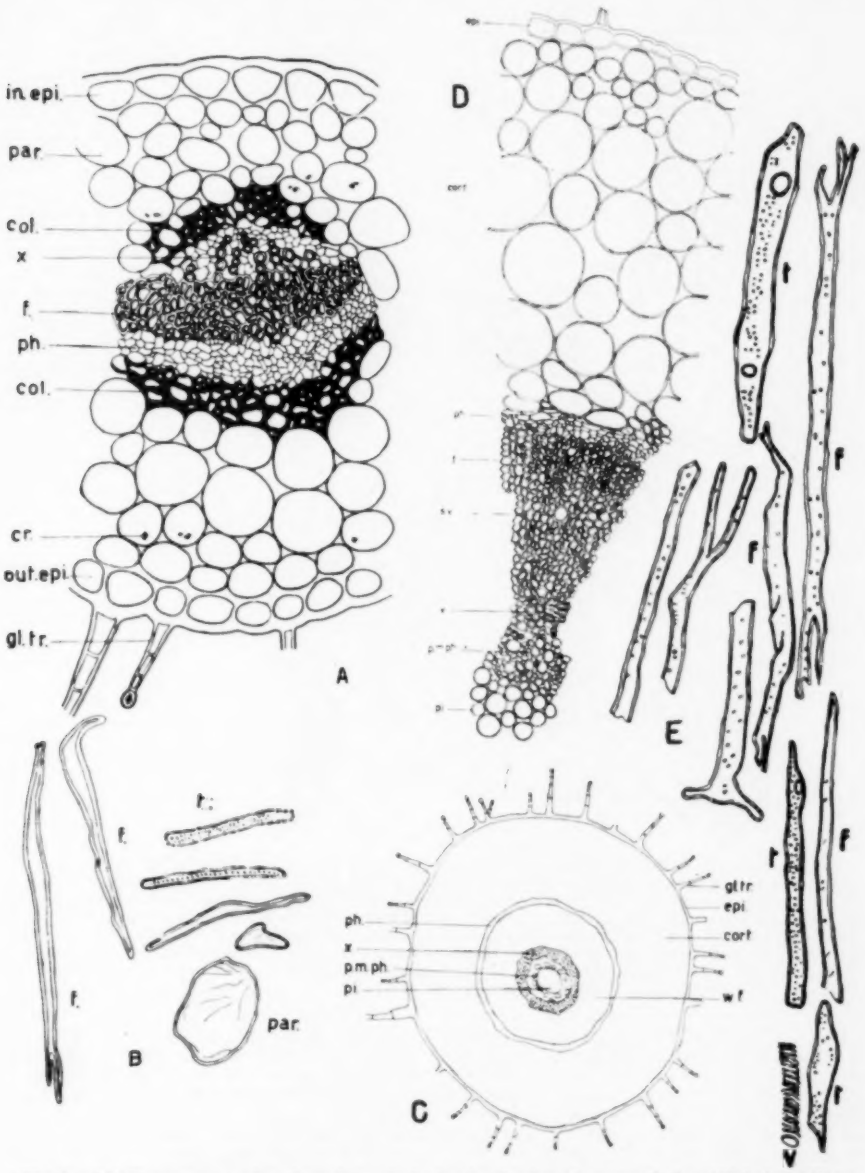

Fig. 2.—(*A*) Transverse section through a principal vein of calyx of mature fruit; (*B*) isolated fibers, tracheids and parenchyma of calyx; (*C*) diagram of T. S. in pedicel of fruit; (*D*) detailed T. S. in pedicel of fruit; (*E*) isolated elements from sclerenchymatous layer of pedicel of fruit; *col.*, collenchyma; *cor.*, cortex; *cr.*, crystals; *epi.*, epidermis; *f.*, fibers; *gl. tr.*, glandular trichrome; *in. epi.*, inner epidermis; *out. epi.*, outer epidermis; *par.*, parenchyma; *ph.*, phloem; *pi.*, pith; *p. m. ph.*, perimedullary phloem; *v.*, vessels; *s. v.*, vessels in sclerenchymatous layer; *w. f.*, wood fibers; *x.*, xylem. (A, B, E, × 150; D × 75; C × 22.)

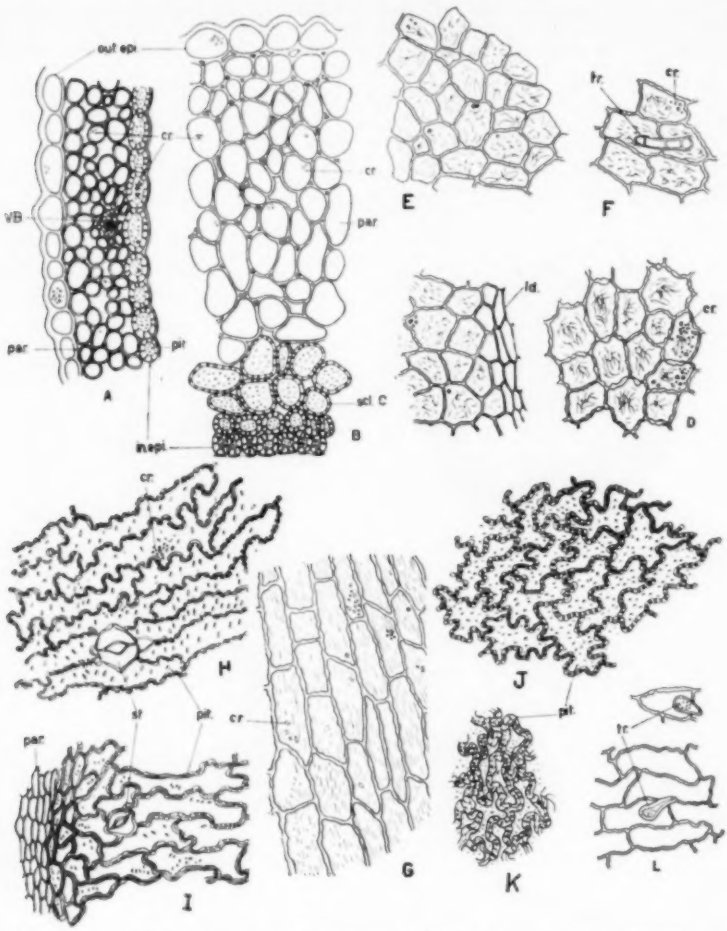

Fig. 3.—Pericarp. (*A*) T. S. of lower part of pericarp; (*B*) T. S. in lid; (*C*) outer epidermis of lid at line of dehiscence; (*D*) outer epidermis of lid showing beadings; (*E*) outer epidermis of lid showing no beading; (*F*) outer epidermis of lower part of lid showing nonglandular trichomes; (*G*) outer epidermis of lower part of fruit; (*H*) inner epidermis of pericarp in lower part of fruit; (*I*) same near line of dehiscence; (*J*) inner epidermis of lid; (*K*) inner epidermis of lid showing no beading; (*L*) basal region of endocarp of pyxis showing nonglandular trichome; *cr.*, crystals; *in. epi.*, inner epidermis; *l. d.*, line of dehiscence; *out. epi.*, outer epidermis; *par.*, parenchyma; *pit.*, pits; *scl.*, sclerenchymatous layer; *st.*, stomata; *tr.*, nonglandular trichomes; *v. b.*, vascular bundle; all ×100.

These cells have thinner straight anticlinal walls, are free from crystals of calcium oxalate, and are 23–84 μ long, 13–40 μ broad, and 33–50 μ high (Fig. 3, *C*). The rest of the outer epidermis of the lid is formed of larger cells which are smaller than those of the lower part of the pericarp, being 43–150 μ long, 27–97 μ broad, and 43–67 μ high.

The outer walls of the epicarpal cells are covered with a rather thick cuticle which is longitudinally striated. They are mostly convex, occasionally dome-like, and in very rare cases they may form short papillae (Fig. 3, *A*, *B*). In the lid, they are distinctly more convex and commonly form low, dome-like structures. The anticlinal walls are straight or slightly curved and usually unequally thickened, occasionally beaded in the lid region (Fig. 3, *D*). The inner tangential walls are equally thickened.

Trichomes are usually absent on the outer epidermis of the pericarp, but may be present rarely in the lid region where they are short, nonglandular, multicellular, uniseriate, with rounded apices, and becoming up to 125 μ long (Fig. 3, *F*).

Calcium oxalate crystals are abundantly present in the outer epidermis, being either isolated or in aggregates. Numerous crystals may be present in

occasional cells. The individual crystal may be either prismatic or cubical, rarely microsphenoidal. They are up to 53 μ long and 23 μ broad. Stomata are very rare on the outer epidermis and are of the cruciferous type, each measuring 32–60 μ long and 32–43 μ broad.

Inner Epidermis (Endocarp).—The endocarp (Fig. 3, *A*, *B*, *G*, *H*, *I*, *K*, *L*) takes the form of a single continuous sclerenchymatous layer formed of polygonal, elongated cells, each with thick strongly lignified walls, a distinct middle lamella, and a very thin cuticle on the innermost walls. The outer and inner tangential walls are usually convex, and the anticlinal walls are unevenly thickened and commonly beaded. The cells vary in size and shape; in the middle region of the pericarp, they are long and axially elongated, being 270–450 μ long, 34–66 μ broad, and 23–44 μ high. Approaching the line of dehiscence, the cells gradually become transversely arranged and measure from 33 to 100 μ long, 17–56 μ broad, and 17–30 μ high (Fig. 3, *I*).

For a distance of about 1 to 3 mm. from the base, the endocarpal cells become nonlignified, with less thickened and less beaded walls. These cells are elongated and irregularly arranged, and measure from 33 to 166 μ long, 30–66 μ broad, and 33–50 μ high. Scattered among these cells are seen islets of similar but lignified cells. A few of the nonlignified cells may protrude into short, unicellular, nonglandular hairs (Fig. 3, *L*).

The endocarpal cells of the lid are similar to those of the lower part of the pericarp, but are all lignified, more wavy, usually more thickened and sometimes not beaded (Fig. 3, *K*). They measure 57–100 μ long, 27–67 μ broad, and 27–40 μ high. But in the region of dehiscence, they are small, transversely elongated parenchymatous cells, with thin, straight, nonlignified walls, and have the dimensions of 20–50 μ long, 10–24 μ broad, and 27–40 μ high (Fig. 3, *I*).

With the exception of the region of dehiscence, all the endocarpal cells are extensively pitted, with slit-like pits which are 3–15 μ long and up to 7 μ broad. Most of these slit-like pits are arranged obliquely or transversely in relation to the direction of the cells. A few small prisms of calcium oxalate are sparsely scattered in the endocarpal cells, especially in the basal part of the fruit.

Nonglandular hairs are found only in the basal regions of the endocarp, being rare and only projecting from nonlignified cells. They are unicellular, conical, slightly curved, and with a round, bent apex, fairly thick lignified walls, a wide lumen, and have pitted lignified bases. They are 50–75 μ long and 30–33 μ broad at the base (Fig. 3, *L*).

A few stomata of the cruciferous type are present in the inner epidermis. They have nonlignified guard cells and measure 46–70 μ long and 40–53 μ broad.

The Mesocarp.—The mesocarp (Fig. 3, *A*, *B*) consists mainly of parenchymatous cells with large and small intercellular spaces. The cells are sometimes rounded, mostly elongated either longitudinally or tangentially, and some of them are branched. They differ in size, being 33–283 μ long, 19–67 μ broad, and 13–50 μ high. The cells become smaller toward the base of the pericarp, where they are 10–53 μ long, 10–30 μ broad, and 10–53 μ high.

In the lid region the mesocarp shows an outer zone of parenchymatous cells, sometimes collenchymatous, with small and large intercellular spaces and containing chloroplasts and sometimes prisms of calcium oxalate. The inner zone is formed of sclerenchymatous cells with thickened, pitted, and lignified walls (Fig. 3, *B*). The sclerenchymatous cells of the mesocarp vary in shape, being round, oval, rectangular, or irregularly elongated, and having simple or branched distinct pits and strongly lignified walls. They also vary in size from 25–200 μ long, 10–117 μ broad, and 14–67 μ high, the biggest cells being toward the outside, with the smallest, thicker-walled cells being toward the inner epidermis, where they are mostly similar to the endocarpal cells.

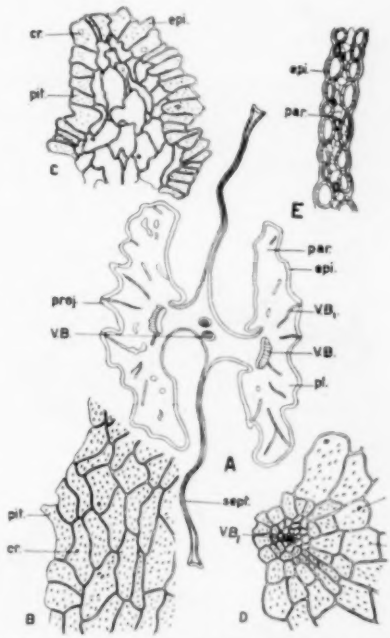

Fig. 4.—(*A*) Diagram of T. S. of septum and placenta, × 27; (*B*) epidermis of placenta in surface view; (*C*) T. S. of placenta; (*D*) epidermis of placenta in surface view at place of attachment of a seed; (*E*) T. S. of septum; *cr.*, crystals; *epi.*, epidermis; *par.*, parenchyma; *pit.*, pits; *pl.*, placenta; *proj.*, projections to which the seeds are attached; *sept.*, septum; *V. B.*, vascular bundles running toward the seeds; all ×100.

Vascular System.—Numerous vascular bundles course through the mesocarp from base to apex, and some of them occasionally give off branches which run upward. In the lid region the vascular bundles may abut upon the sclerenchymatous layer. The bundles are slender, up to 100 μ in diameter, collateral, with the xylem toward the inner side and the phloem toward the outer surface. The xylem is composed of vessels, mostly spiral

and 7–10 μ in diameter, a few tracheids 60–117 μ in length and 13–17 μ in diameter, and occasional fibers 117–730 μ in length.

The Septum.—The septum (Fig. 1, *G, H;* Fig. 4, *A, E*) consists of two epidermises which are continuous with the inner epidermis of the pericarp, enclosing between them one or a few layers of comparatively very small parenchyma having thin sinuous walls. The parenchymatous layer varies in thickness, being from 3 to 4 cells thick in the middle, 7 to 10 cells where the septum joins the pericarp, and up to 8 cells thick where the septum joins the placenta.

The epidermal cells on both sides are lignified and are identical both in size and shape with those of the inner epidermis of the pericarp.

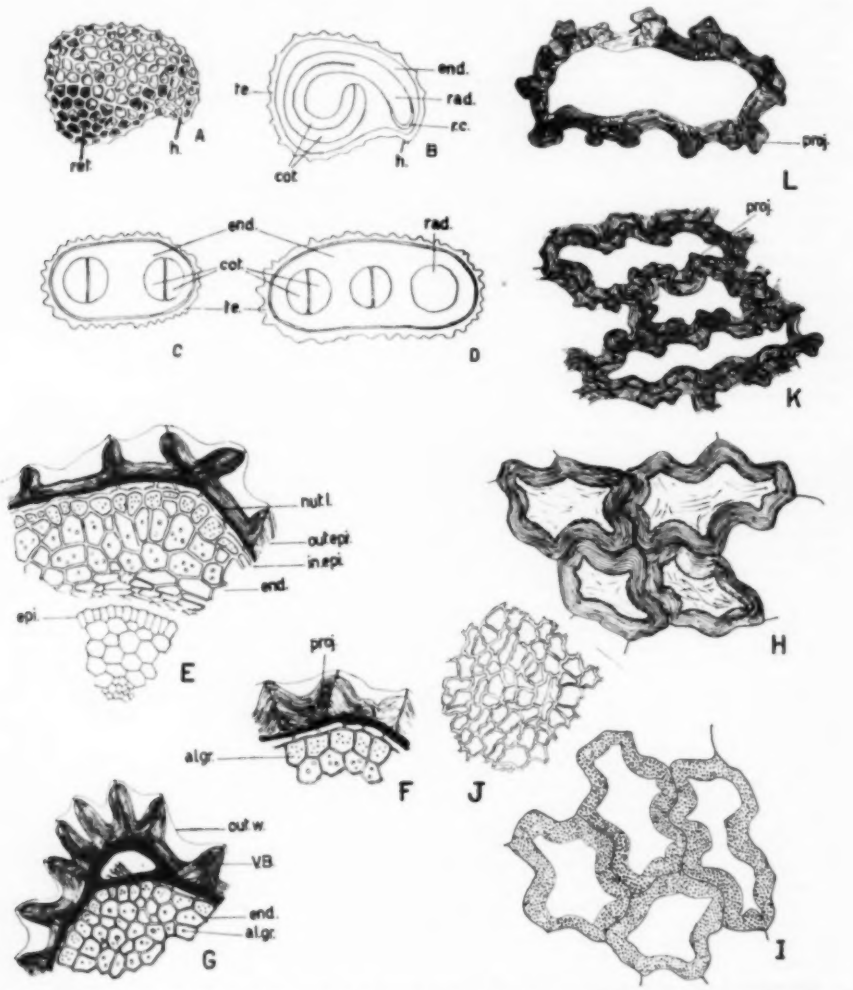

Fig. 5.—Seed. (*A*) Entire seed, × 18; (*B*) diagram of L. S. parallel to flat surface, × 18; (*C*) diagram of T. S. of seed, × 25; (*D*) diagram of L. S. perpendicular to flat surface, × 25; (*E*) and (*F*) T. S. in seed; (*G*) T. S. in seed passing through raphe; (*H*) Outer epidermis of testa, focused on the middle of anticlinal walls; (*I*) outer epidermis of testa, focused on the upper part of the thickened anticlinal walls; (*J*) inner epidermis of testa; (*K*) few cells of outer epidermis of testa showing projections protruding into the neighboring walls; (*L*) isolated cell of outer epidermis of testa showing projections from walls; *E, F, G, H, I, J, K* and *L* ×125; *al. gr.*, aleurone grains; *cot.*, cotyledons; *end.*, endosperm; *epi.*, epidermis of radicle; *h.*, hilum; *in. epi.*, inner epidermis of testa; *nut. l.*, nutritive layer; *out. epi.*, outer epidermis of testa; *out. w.*, outer walls of outer epidermal cells; *proj.*, projections; *rad.*, radicle; *r. c.*, root-cap; *ret.*, reticulations; *te.*, testa; *V. B.*, vascular bundle.

The Placenta.—The ground tissue of the placenta (Fig. 1, *F, G;* Fig. 4, *A, B, C, D*) is formed of irregular parenchyma, with thin nonlignified walls and wide intercellular spaces (Fig. 4, *C*). The epidermal cells are polygonal, appearing elongated and irregular in surface view (Fig. 4, *B*) and more or less rectangular in a cross section (Fig. 4, *C*), and being 27–156 μ long, 66–100 μ broad, and 33–80μ high. The walls of the epidermal cells are thin, slightly wavy, pitted, and lignified.

Prisms of calcium oxalate are seen in some of the parenchymatous cells and occasionally in the epidermal cells.

Three to six main vascular bundles traverse the placenta vertically and bear numerous side branches running toward the attached seeds (Fig. 1, *F*).

The surface of the placenta exhibits projections in the form of large papillae to which the seeds are attached (cicatrices) (Fig. 4, *D*).

MACROMORPHOLOGY OF SEED

The seeds of *Hyoscyamus muticus* L. are yellowish-grey to brown in color. They are more or less reniform and triangular, sometimes elongated, having a broad end and a narrow end which tapers into a beak, more or less short and pointed. The seeds are laterally compressed, 1–2 mm. long, 1–2 mm. broad, and 0.5 to 1.5 mm. in thickness. They are odorless and possess an oily bitter taste. The seeds are easily crushed under the teeth.

Externally the seed is finely reticulated; the reticulations are polygonal with wavy sides surrounding shallow depressions. The number of reticulations across the flat side of the seed is 8 to 12, while there are 9 to 13 reticulations lengthwise. The hilum is represented by a small, narrow, slightly depressed scar, situated in the narrow pointed end of the seed (Fig. 5, *A*).

Internally the seed consists of a thin testa enclosing a copious oily endosperm in which is embedded a coiled embryo, with the radicle pointed toward the hilum and the two cotyledons boldly curved or almost coiled, while the small hypocotyle lies between the radicle and the cotyledons (Fig. 5, *B, C, D*).

MICROMORPHOLOGY OF SEED

The testa consists of an outer epidermis, followed by a nutrient layer of brownish collapsed cells, and limited on the inner side by the inner epidermis of the integuments (Fig. 5, *E, F, G*).

The outer epidermis of the testa consists of cells which in surface view appear polygonal, elongated, or isodiametric, and have wavy walls (Fig. 5, *H, I*). In transverse section, they appear more or less rectangular or triangular, especially in the edges of the seed. The epidermal cells are 40–350 μ long, 36–116 μ broad, and 43–83 μ high. The outer tangential walls are thin, cellulosic, transparent, and covered with a thin cuticle. They are usually concave, occasionally flat, but sometimes waxy and completely depressed into the cell cavity. In certain cases, the depression is so deep that the outer wall comes very near to, or may even touch, the inner tangential wall, which is very thick, distinctly striated, slightly lignified, and flat, curved, or wavy on the inner side. The outer part of the anticlinal wall is thin, cellulosic, straight or rarely wavy, while the rest of the wall is abruptly and distinctly thickened and striated, the thickening and striations being similar to, and continuous with, those of the inner tangential wall.

The inner or free surface of the thickened part of the cell wall, i. e., the surface lining the cavity of the cell, is not smooth but appears warty due to the presence of numerous minute nodules (Fig. 5, *I*).

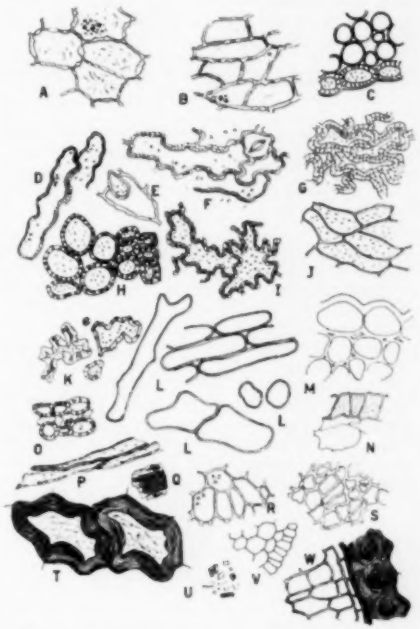

Fig. 6.—Powdered fruits. (*A*) Outer epidermis of lid; (*B*) outer epidermis of pericarp; (*C*) fragment of pericarp showing section in endocarp and mesocarp; (*D*) endocarpal cells; (*E*) inner epidermis from pericarp of lower part of fruit showing nonglandular trichome; (*F*) endocarp of lower part of fruit showing stoma; (*G*) endocarp of lid; (*H*) fragment of lid showing endocarp and sclerenchymatous layer; (*I*) endocarp of lid; (*J*) epidermis of placenta; (*K*) cells from inner region of mesocarp of lid; (*L*) parenchyma of pericarp; (*M*) fragment of lid; (*N*) epidermis of placenta in sectional view; (*O*) fragment of septum; (*P*) group of fibers from pericarp; (*Q*) spiral vessels from pericarp; (*R*) endosperm cells from seed; (*S*) inner epidermis of testa; (*T*) fragment of radicle in sectional view; (*U*) calcium oxalate crystals; (*V*) fragment of seed in sectional view; all ×100.

From the thickened part of the anticlinal walls, projections of variable shape and size protrude into the neighboring walls and thus greatly reinforce the testa (Fig. 5, *K, L*).

The nutrient layer is brown in color, about 10 μ thick, and is formed of collapsed cells.

The inner epidermis of the testa is formed of a single layer of 4- to 6-sided elongated cells, with straight or curved walls (Fig. 5, *J*). They are

collapsed and thus indistinct in certain areas. These cells are 30–57 μ long, 17–37 μ broad, and 3–13 μ high.

The endosperm is formed of polygonal, usually 5- to 6-sided, occasionally rectangular cells, with non-lignified, moderately thick walls. The cells contain fixed oil and small aleurone grains, 2–10 μ in diameter; each aleurone grain usually contains a crystalloid and a globoid.

The cells of the embryo are small, polygonal, and have very thin cellulosic walls, and also contain aleurone grains (Fig. 5, *E*) similar to those of the endosperm.

POWDERED FRUIT OF *HYOSCYAMUS MUTICUS*

The ripe fruit of *Hyoscyamus muticus* L., when dried and reduced to a No. 90 powder, is greenish- or yellowish-brown, with an unpleasant odor. This powder is identified by the following structures, which are arranged in order of their importance:

1. Inner epidermis of pyxidium and inner zone of sclerenchymatous layer of lid; fragments of this tissue are present in section or surface views, showing polygonal elongated cells with unevenly thickened, commonly beaded, strongly lignified wavy walls, with those of the lid usually more wavy and thickened (Fig. 6, *C, D, F, G, H, I, K*).

2. Outer epidermal cells of the seed-coat which appear, in surface view, polygonal, elongated, or isodiametric, with wavy walls, and in sectional view appear more or less rectangular or triangular, with thin cellulosic outer tangential walls and slightly lignified, distinctly striated, much thickened brown inner and anticlinal walls, with rounded projections and wartiness (Fig. 6, *T, W*).

3. Endosperm cells which are polygonal or rectangular, cellulosic and containing fixed oil and aleurone grains, 2–10 μ in diameter, each composed of a crystalloid and a globoid (Fig. 6, *R, W*).

4. Outer epidermis of the pericarp showing polygonal cells, with unequally thickened anticlinal walls and a thick striated cuticle, the striations appearing as longitudinal parallel streaks or radiating from the center in the cells from the lid region, where the walls are occasionally beaded (Fig. 6, *A*). Occasional cells contain calcium oxalate prisms (Fig. 6, *A, B, M*).

5. Epidermal cells of the placenta with thin, not straight, pitted and lignified walls, occasionally with calcium oxalate crystals (Fig. 6, *N, J*).

6. Occasional fibers and slender vessels, mostly with spiral thickening, from the mesocarp (Fig. 6, *P, Q*).

7. Parenchyma cells of mesocarp and placenta (Fig. 6, *L*).

8. Calcium oxalate crystals, few, occasionally free, mostly prisms or small clusters, very rarely microsphenoidal, up to 53 μ long and 23 μ broad (Fig. 6, *U*).

REFERENCES

(1) Fahmy, I. R., *Repts. Pharm. Soc. Egypt*, **2**, 1(1931).
(2) Wallis, T. E., "Textbook of Pharmacognosy," 1st ed., J. & A. Churchill, Ltd., London, 1946.
(3) Ahmed, Z. F., thesis presented to the Faculty of Medicine, Kasr El-Ainy, Fouad I University, 1944, for the degree of Master of Science in Pharmacy.
(4) Saber, A. H., and Balbaa, S. I., *Repts. Pharm. Soc. Egypt*, **33**, 29 (1951).
(5) Rohatgi, S., and Fairbairn, J. W., *J. Pharm. Pharmacol.*, **2**, 286(1950).

Use of the Range in the Statistical Evaluation of a Biological Assay*

By LILA KNUDSEN RANDOLPH†

The statistical formulas used in the biological assay of digitalis, ouabain, and tubocurarine chloride in U. S. P. XIV are explained and short-cut procedures developed. These simplified procedures are based upon the principle that for the number of animals usually employed in the assays, the range (the highest value minus the lower value) is as good an index to the standard error as the standard deviation and is much more easily calculated.

In U. S. P. XIV (1) certain biological assays have been modified to allow for a statistical estimation of potency and of the standard error of the assay. These include the assays for digitalis tincture, lanatoside C injection, ouabain injection, testosterone propionate injection, and tubocurarine chloride injection. The Pharmacopeia provides short-cut procedures for the calculations (2) involved in these assays. It is the purpose of this paper to derive and explain these rapid statistical methods, particularly

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† Consultant, Food and Drug Administration, Federal Security Agency, Washington, D. C.

the use of the range in the formulas for the standard error of the assay. (The range is the highest response minus the lowest response.)

STANDARD ERROR OF THE ASSAY

Before deriving the short-cut methods, it may be wise to make some explanation of the standard error of the assay. Usually the standard error of a statistic can be used to calculate limits within which future estimates of that statistic will occur with a specified probability. Thus some multiple of the standard error of the assay is frequently used to calculate so called "confidence limits for the potency"—limits within which potencies indicated by other assays on the same substance will occur, say, 19 times in 20 ($P = 0.95$). However, the formula for calculating the standard error of an assay can only reflect causes of variation that influence the results of the initial assay. There may be other factors that are constant at one time or in one laboratory but that vary from time to time or from laboratory to laboratory. Such factors would therefore not enter into the standard error of the assay, as calculated from the variation within an assay. Thus the confidence limits calculated from the standard error of the assay may be erroneous as applied to repeated assays. The use of the standard error of the assay (as calculated from the internal variation of a single assay) is explained somewhat in a sentence on page 689 of U. S. P. XIV (1) wherein it states, "While at present it [the standard error of the assay] is the best index to the extent of these variations, [between several estimates of potency] it may not measure all of the factors leading to differences in the indicated potency of a given preparation arising between laboratories or even between successive assays in the same laboratory." This serves to emphasize that any approximate limits of the potency, calculated through use of the standard error of the assay, are not necessarily the bounds within which would fall a specified proportion of estimates of the potency from other assays. In U. S. P. XIV the standard error of the assay is used merely as one of the possible measures of the reliability of a particular assay. In a recent article (3), several collaborative studies involving biological assays have been analyzed in order to see whether the standard error of the assay is a good index of reproducibility of the estimate of a potency. It was concluded that in many of the assays now official, the confidence limits computed from the standard error of the assay were too small.

The usual statistical formulas for the standard error of the assay involve the calculation of either the standard deviation of a series of responses to the same dose of a drug, the standard error of estimate about a regression line, or some modification of either of these. It has been possible to simplify some of these calculations through use of short-cut formulas such as given in U. S. P. XIV by employing the range instead of the standard deviation or standard error of estimate.

THE RANGE

The range has a definite relationship to the standard deviation depending on the number of observations, and has the advantage of being much simpler to calculate than is the standard deviation. Tippett (4) has shown that the average range is a constant multiple "k" of the average standard deviation for a given sample size (number of animals on a given dose, in the case of biological assay). In the last few years much has been written on the use of the range in "quick and dirty" statistical methods (5–8) and also on the use of the range in biological assay (9).

Simon (10), in writing about the use of the range as a substitute for the standard deviation states that there is a marked loss of efficiency of the range as compared to the standard deviation as the sample size increases. "However as the sample size decreases, the disparity in efficiency also decreases; and of course the (two) statistics are equally efficient for $n = 2$. For small samples ($n = 2$ to $n = 10$ or 15) the loss in efficiency is relatively slight." He compares the precision of the range and the standard deviation in a graph (11) showing the required sample size for equal precision using the range, on the one hand, and the standard deviation on the other. Table I gives this information.

However, some work being done by Mosteller and Berkson (12), gives evidence that the range is much better than has been supposed even for large samples. In fact, it appears that although the efficiency of the range decreases with increasing sample size, other factors improve. The resulting conclusions will be published shortly, and apparently will redeem the range from its banishment for use with large samples.

TABLE I.—COMPARATIVE SIZE OF SAMPLES FOR EQUAL PRECISION[a]

Using Range	Using Standard Deviation
n	n
2	2
5	5
9	8
15	12
20	14
etc.	etc.

[a] From the original graph by Simon.

DIGITALIS AND OUABAIN

The formula for the potency in assays for digitalis tincture and ouabain is essentially the dilution factor multiplied by the ratio of the average number of doses of standard to the average number of doses of unknown necessary to produce cardiac arrest in pigeons.

P = Potency in U. S. P. digitalis units per cc.

$$P = \frac{\bar{y}_s \times \text{No. of cc. of standard preparation in 100 cc. of test dilution}}{\bar{y}_u \times \text{No. of cc. of preparation to be assayed in 100 cc. of test dilution}}$$

where $\bar{y}_s$ is the average number of doses of standard test dilution which were injected and $\bar{y}_u$ is the average number of doses of the unknown injected.

The formula for the standard error of the assay is essentially the formula for the standard error of the ratio $\bar{y}_s/\bar{y}_u$ which is derived according to the theory of errors from

$$\frac{\sigma^2_P}{P^2} = \frac{\sigma^2_{\bar{y}_s}}{(\bar{y}_s)^2} + \frac{\sigma^2_{\bar{y}_u}}{(\bar{y}_u)^2}$$

However,

$$\sigma^2_{\bar{y}_s} = \frac{\sigma^2_{y_s}}{N} = \frac{R^2_s}{k^2 N}$$

letting

$$g = \frac{1}{k^2 N}$$

then

$$\sigma^2_{\bar{y}_s} = g_s R^2_s$$

also

$$\sigma^2_{\bar{y}_u} = g_u R^2_u$$

Substituting and transposing yields the formula for the standard error of the assay given in the U. S. P. XIV:

standard error of the assay =

$$\text{Potency} \sqrt{g_s \frac{(R_s)^2}{(\bar{y}_s)^2} + g_u \frac{(R_u)^2}{(\bar{y}_u)^2}}$$

In the above derivation, $\sigma_{\bar{y}_s}$ is the standard error of the average number of doses of standard injected into each pigeon. R_s is the range of the number of doses of the test dilution of the standard which were injected, and is obtained by subtracting the smallest number of doses from the largest. The value "N" indicates the number of pigeons injected with each test dilution and "k"[1] is the average number of standard deviation units encompassed by the average range, as obtained from a table worked out by Tippett (4). Since the value of "k" is dependent solely on N, and the value of g involves only k and N, values of g corresponding to N were calculated and a table of these values was inserted in the assay procedure as given in U. S. P. XIV. Since N_s, the number of pigeons used on the standard, can differ from N_u the number used on the unknown, two values of g, g_s, and g_u, are provided for in the formula.

LANATOSIDE C INJECTION

The formula for the standard error of the assay is essentially the same as that for digitalis. Again the value of g is the same:

$$g = \frac{1}{k^2 N}$$

TUBOCURARINE CHLORIDE INJECTION

In the tubocurarine chloride assay suitable rabbits are injected with a constant dose of either standard or unknown solution periodically until head-drop occurs. The potency is a function of the number of doses necessary for head-drop. The rabbits are divided into two equal groups of at least eight each. One group is injected first with the standard and subsequently with the unknown, while the second group receives the unknown first and then the standard. The number of doses necessary to produce head-drop in each animal is recorded. For each animal the ratio of the number of doses of the standard to the number of doses of the unknown is calculated and the logarithm of this ratio is found. The potency involves the sum of the logarithms for the first group plus the sum of the logarithms for the second group. The standard error of the assay then is essentially based on the standard error of the average for two groups. Thus

standard error of the assay =

$$2.3 \times \text{potency} \times \sqrt{\frac{N_1\sigma_1^2 + N_2\sigma_2^2}{(N_1 + N_2)^2}}$$

Substituting $\sigma = \frac{R}{k}$ and assuming $N_1 = N_2 = N$, since the two groups have an equal number of animals due to the crossover design of the assay, the

$$\text{standard error of assay} = \frac{2.3}{2k\sqrt{2N}} \times \text{potency} \times (R_1 + R_2)$$

Thus we obtain the formulas as given in U. S. P. XIV:

standard error of assay = $c \times$ potency $\times (R_1 + R_2)$

where the value of $c = \frac{2.3}{2k\sqrt{2N}}$ is tabled on page 647 of U. S. P. XIV.

TESTOSTERONE PROPIONATE

The formula for the standard error of the assay for testosterone propionate is essentially the same as that derived for the penicillin assay (9) except that R is the sum of the four ranges in response for the two dosage levels of the unknown and of the standard. Again k is the average number of standard deviation units in the average range.

REFERENCES

(1) "United States Pharmacopeia," Fourteenth Revision, Mack Publishing Co., Easton, Pa., 1950.
(2) Munch, J. C., Kuna, S., and Dietrick, E. V., *Am. J. Pharm. Ed.*, **15**, 1(1951).
(3) Knudsen, L. F., "A Comparison of Standard Errors from Collaborative Bioassays," in press.
(4) Tippett, L. H. C., *Biometrika*, **17**, 364(1925).
(5) Knudsen, L. F., *J. Am. Statistics Assoc.*, **38**, 466 (1943).
(6) Daly, J., *Ann. Math. Statistics*, **17**, 71(1946).
(7) Lord, E., *Biometrika*, **37**, 64(1950).
(8) Mosteller, F., and Tukey, J. W., *Ind. Quality Control*, **6**, 5(1949).
(9) Knudsen, L. F., and Randall, W. A., *J. Bact.*, **50**, 187(1945).
(10) Simon, L. E., "An Engineers Manual of Statistical Methods," John Wiley & Sons, Inc., New York, 1941, p. 139.
(11) Simon, L. E., *ibid.*, p. 135.
(12) Mosteller, F., and Berkson, J., "Another Viewpoint on the Efficiency of the Range in Large Samples," presented at the annual convention of the American Statistical Association, Boston, Mass., December, 1951.
(13) Pearson, K., "Tables for Statisticians and Biometricians," 1st ed., Cambridge University Press, London, 1931, p. 165.

[1] This is the same value of "k" as given in U. S. P. XIV in the formulas for the standard error of the assay for testosterone propionate injection. The value of k is tabled in various statistical texts and in Pearson's tables (13).

State of Subdivision. V. Magnitude and Variations in Specific Surface Areas*

By J. V. SWINTOSKY and A. NARSIMHA RAO

In recent years interest has increased in the effect of particle sizes and specific surface areas of pharmaceutical powders and the way in which such parameters influence the degree of therapeutic action and the pharmaceutical technology of these products. A number of lots of different powders obtained from various sources were subjected to nitrogen adsorption studies to determine the relative average particle size of each chemical substance in its present commercial forms and the uniformity of specific surface area of these powders from lot to lot. Data are presented for measurements on zinc oxide, barium sulfate, ammoniated mercury, bismuth subnitrate, bismuth subcarbonate, and sulfathiazole. More precise particle size and surface area specifications for pharmaceutical powders are recommended.

THE U. S. P. and N. F. monographs do not include any fineness specifications for some of the official powders. For others the specifications of fineness are expressed only in simple descriptive terms such as a very coarse powder, a coarse powder, a moderately coarse powder, a fine powder, or a very fine powder. These descriptive terms are in turn related to a number assigned to a certain standard sieve. Such terms may be adequate for describing powders derived from crude drugs; but for most pure chemical powders used in pharmacy these terms are inadequate, because the powders under consideration possess degrees of fineness which fall outside the sieve size range. These powders thus become indistinguishable, with respect to particle size, by conventional sieve analysis. Since there appear to be significant relationships between the surface area and particle size characteristics of some powders, and their physiologic activities (1), and since fineness characteristics and deviations from uniformity of these characteristics for a given powdered substance affect the technology of pharmaceuticals prepared from it, it seemed desirable to (*a*) determine the average particle size of each of several lots of different official chemical powders; and (*b*) investigate the various lots of several official powders with the objective of obtaining precise specific surface area values for comparison of average particle size from lot to lot. Such data would be necessary to establish whether, for a given powder, present control methods are satisfactory for the production of products uniform with regard to the state of subdivision. Also, data on the relative particle size of chemically different powders would be useful in the scientific approach to the solution of technologic problems arising from their pharmaceutic use.

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EXPERIMENTAL

Powders Studied.—The powders chosen for this investigation were barium sulfate U. S. P., zinc oxide U. S. P., sulfathiazole U. S. P., ammoniated mercury U. S. P., bismuth subcarbonate U. S. P., and bismuth subnitrate N. F. These powders were chosen because of their common usage in pharmacy, their ready availability, and their amenability to analysis.

Procedure.—The specific surface areas were determined by low-temperature nitrogen adsorption (BET method), a method which has seen previous application in similar studies on pharmaceutical powders (2–5). This procedure, and its development by other investigators, has been described in a previous paper (2); however, several minor modifications in the present procedure deserve mention. Experience has shown that for the precision desired, the vapor pressure of the liquid nitrogen, P_0, did not vary sufficiently to warrant redetermination for every surface area analysis. Therefore, 70.5 cm. was taken as an average constant value for P_0 with consequent simplification of the experimental runs. Also, it was observed that the specific surface area values obtained by drying and degassing of samples at elevated temperatures did not vary appreciably from values obtained by degassing at room temperature. Thus, for all samples, degassing was conducted under high vacuum overnight at room temperature. The adsorption isotherms were obtained in the same manner as described in a previous paper (2), by substituting the appropriate values in the BET equation

$$\frac{P}{V(P_0 - P)} = \frac{1}{V_m C} + \frac{(C-1)P}{V_m C P_0}$$

RESULTS AND DISCUSSION

Results of the analyses are shown in Table I and Figs. 1–6. The various samples have been designated in code number by using a single alphabetical letter to indicate the company supplying the product, a roman numeral to indicate the lot number of the product, and an arabic numeral to indicate the analysis number. Thus, the first powder in Table I, designated A-I-1, was supplied by Company A; it was a lot designated No. I; and the experimental specific surface area corresponding to this code number was the result of our analysis No. 1.

TABLE I.—SURFACE AREA MEASUREMENTS ON VARIOUS LOTS OF DIFFERENT POWDERS DETERMINED BY LOW TEMPERATURE NITROGEN ADSORPTION

Material	Code No.	Specific Surface Area, m^2/Gm.	Average Area for Each Lot, m^2/Gm.	Density	Surface Average Diameter of Particle, μ
Zinc Oxide U. S. P.	A-I-1	3.99	3.98	5.47	0.28
	2	3.96			
	A-II-1	4.08	4.09		0.27
	2	4.09			
	B-I-1	3.96	3.97		0.28
	2	3.97			
	B-II-1	3.75	3.77		0.29
	2	3.77			
	3	3.79			
	B-III-1	3.55	3.56		0.31
	2	3.55			
	3	3.61			
	4	3.52			
	C-I-1	4.28	4.27		0.26
	2	4.25			
	C-II-1	3.81	3.80		0.29
	2	3.78			
Barium Sulfate U. S. P.	A-I-1	2.32	2.32	4.50	0.58
	2	2.32			
	B-I-1	2.00[a]	2.36		0.60
	2	2.40			
	3	2.32			
	B-II-1	2.68	2.52		0.53
	2	2.48			
	3	2.55			
	4	2.36			
	C-I-1	2.14	2.12		0.63
	2	2.10			
	C-II-1	2.54	2.56		0.52
	2	2.57			
Ammoniated Mercury U. S. P.	B-I-1	0.93	0.96	5.27	1.19
	2	0.99			
	C-I-1	0.58	0.59		1.93
	2	0.60			
Bismuth Subnitrate U. S. P.	A-I-1	0.74	0.75	4.93	1.62
	2	0.75			
	A-II-1	0.47	0.47		2.49
	2	0.47			
	3	0.47			
	B-I-1	0.50	0.58		2.10
	2	0.56			
	3	0.69			
	B-II-1	0.79	0.76		1.60
	2	0.75			
	3	0.72			
	B-III-1	0.59	0.63		1.93
	2	0.67			
	3	0.63			
	C-I-1	0.27	0.27		4.51
	2	0.27			
	C-II-1	0.39	0.40		3.04
	2	0.40			
Bismuth Subcarbonate U. S. P.	A-I-1	7.55	7.46	6.86	0.12
	2	7.37			
	A-II-1	8.06	7.90		0.11
	2	7.73			
	B-I-1	4.62	4.54		0.19
	2	4.45			
	B-II-1	4.96	4.95		0.18
	2	4.94			
	B-III-1	4.83	4.80		0.18
	2	4.76			
	C-I-1	6.55	6.70		0.13
	2	6.79			
	3	6.77			
	C-II-1	7.13	7.13		0.12
	2	7.13			

(*Continued on p. 443*)

TABLE I.—SURFACE AREA MEASUREMENTS ON VARIOUS LOTS OF DIFFERENT POWDERS DETERMINED BY LOW TEMPERATURE NITROGEN ADSORPTION (*Continued*)

Material	Code No.	Specific Surface Area, m^2/Gm.	Average Area for Each Lot, m^2/Gm.	Density	Surface Average Diameter of Particle, μ
Sulfathiazole U. S. P.	A-I-1	0.82	0.80	1.57	4.78
	2	0.77			
	A-II-1	0.49	0.49		7.80
	2	0.49			
	B-I-1	0.72	0.72		5.31
	2	0.72			
	B-II-1	0.71	0.71		5.39
	2	0.70			
	B-III-1	0.43	0.43		8.79
	2	0.43			

[a] Value not included in average.

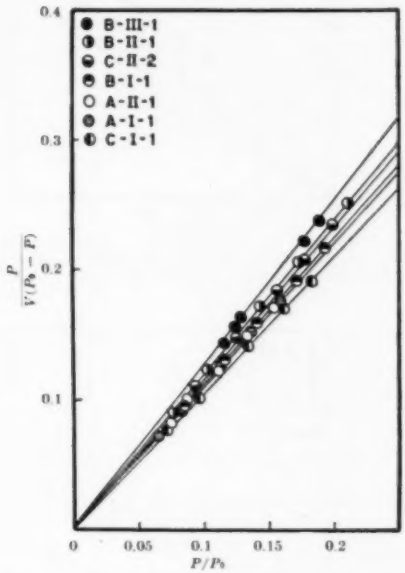

Fig. 1.—Adsorption data for nitrogen on zinc oxide, plotted according to the BET equation.

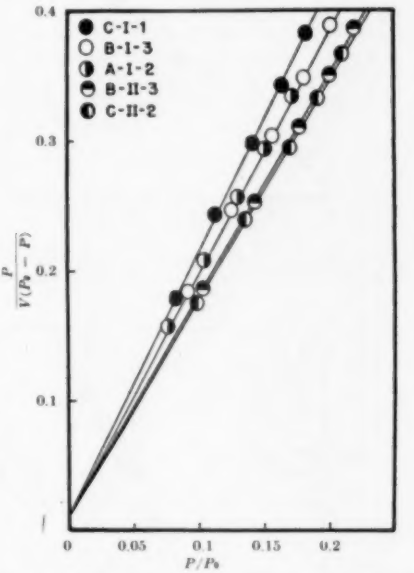

Fig. 2.—Adsorption data for nitrogen on barium sulfate, plotted according to the BET equation.

The data in Table I indicate a considerable variation in surface average particle size from one chemical substance to another. Bismuth subcarbonate and zinc oxide are examples of powders which have been shown to possess very fine textures. Sulfathiazole and bismuth subnitrate show relatively coarse textures. The other powders studied possess particle sizes intermediate between zinc oxide and sulfathiazole. It might be surmised that the usual commercial zinc oxide powders would be particularly adapted to the preparation of smooth and uniform ointments and suspensions. This is borne out in practice. The usual sulfathiazole powders, on the other hand, might be expected to present problems in the manufacture of smooth and uniform ointments and suspensions, and this supposition is likewise borne out in practice. It would seem apparent, then, that the relatively high degree of fineness observed in the commercial zinc oxide powders would be highly desirable in some of the other powders in order to facilitate the preparation of the most satisfactory types of pharmaceutical products from them.

Of the materials studied, it is also apparent that various lots of a given powder supplied by different manufacturers and distributors show appreciable and readily determinable variations in specific surface area. The best powder in the group studied was zinc oxide, which showed not only a relatively high measure of fineness but also a minimal variation in surface average particle size from lot to lot. Bismuth subnitrate, on the other hand, was shown to possess a relatively coarse texture and exhibited considerable variation in particle size from lot to lot. For example, the samples of this powder labeled B-II and C-I in Table I show an almost three-fold difference. The differences in particle size observed for some of the various lots of individual powders no

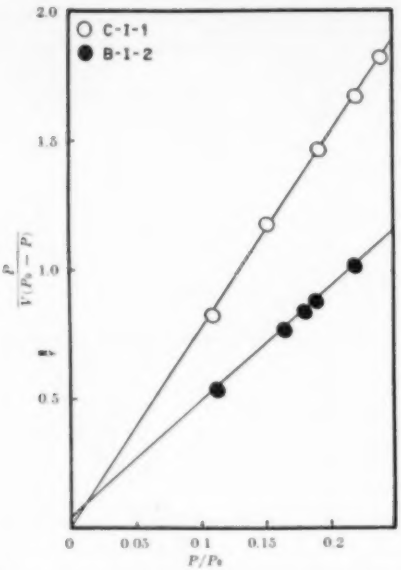

Fig. 3.—Adsorption data for nitrogen on ammoniated mercury plotted according to the BET equation.

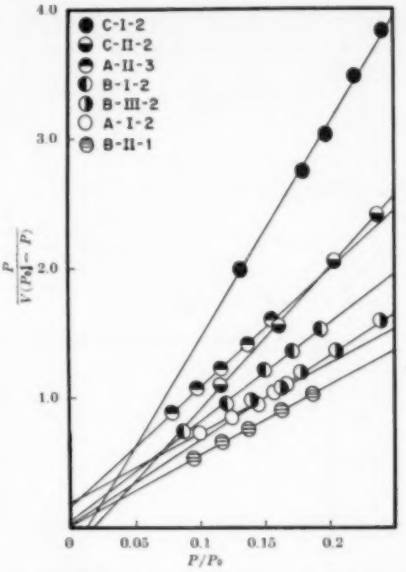

Fig. 4.—Adsorption data for nitrogen on bismuth subnitrate plotted according to the BET equation.

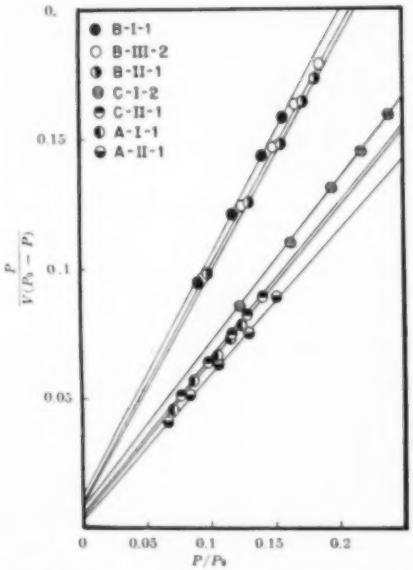

Fig. 5.—Adsorption data for nitrogen on bismuth subcarbonate plotted according to the BET equation.

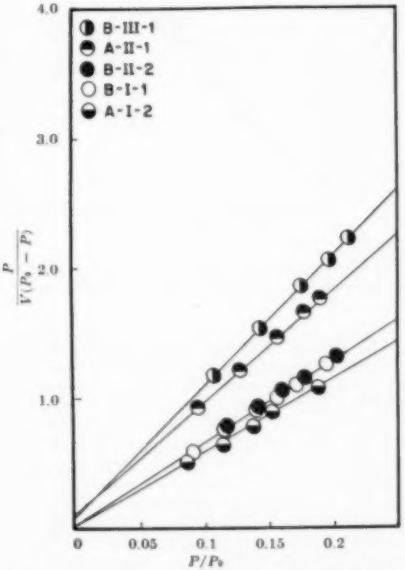

Fig. 6.—Adsorption data for nitrogen on sulfathiazole plotted according to the BET equation.

doubt would be reflected in differences in the settling rates of suspensions and possibly might be reflected in other ways in other types of preparations, such as ointments and dusting powders, derived from these materials. It seems unlikely, however, that the differences observed in the particular samples

studied would markedly influence the degree of therapeutic activity for a given weight of the powder.

Analyses of these powders indicate that the differences in specific surface area for separate lots of any of the chemical substances studied did not exceed threefold variation; however, studies in these laboratories and others indicate that, for many substances, it is a relatively easy matter to prepare powders of a wide range of size and surface specifications. These characters, when appreciably different, affect the technology employed in preparing stable and acceptable pharmaceutical products. Also the physiologic effect of drugs is quantitatively affected by these properties. With methods now available to prepare powders of different size specifications and to conveniently measure these specifications, it would seem that further studies following these lines should be pursued. The objectives of these studies should be the extension of specifications of fineness and uniformity for different powders with which it would be practical for the chemical manufacturers to comply; yet the specifications should be such as to overcome the major shortcomings of some of the currently available powders.

SUMMARY

A comparison of the specific surface areas of various lots of pharmaceutical powders from different companies is illustrated in data for zinc oxide, barium sulfate, ammoniated mercury, bismuth subnitrate, bismuth subcarbonate, and sulfathiazole. Average values expressed in square meters per gram, found for different lots of the respective powders, were as follows: Zinc oxide: 3.98, 4.09, 3.97, 3.77, 3.56, 4.27, 3.80; barium sulfate: 2.32, 2.36, 2.52, 2.12, 2.56; ammoniated mercury: 0.96, 0.59; bismuth subnitrate: 0.75, 0.47, 0.58, 0.76, 0.63, 0.27, 0.40; bismuth subcarbonate: 7.46, 7.90, 4.54, 4.95, 4.80, 6.70, 7.13; sulfathiazole: 0.80, 0.49, 0.72, 0.71, 0.43. The several lots of the single powders analyzed show readily measurable differences in specific surface area. Since it is now possible to measure specific surface areas and particle sizes of many chemical substances with a high degree of precision, it would seem that for such official substances more exacting specifications pertaining to such parameters should be adopted.

REFERENCES

(1) Lyman, R., "American Pharmacy," Vol. 2, J. B. Lippincott Co., Philadelphia, 1947, pp. 210–212.
(2) Swintosky, J. V., Riegelman, S., Higuchi, T., and Busse, L. W., THIS JOURNAL, **38**, 210(1949).
(3) Swintosky, J. V., *et al.*, *ibid.*, **38**, 308(1949).
(4) Swintosky, J. V., *et al.*, *ibid.*, **38**, 378(1949).
(5) Riegelman, S., Swintosky, J. V., Higuchi, T., and Busse, L. W., *ibid.*, **39** 444(1950).

Antitubercular Agent Formed by Condensing Bis (4-Aminophenyl)-Sulfone with L-Ascorbic Acid*,†

By W. D. KUMLER and PETER P. T. SAH

The compound formed by condensing L-ascorbic acid with bis(4-aminophenyl)-sulfone has high *in vivo* activity against tuberculosis. Tests on animals and humans indicate that it is one of the most effective sulfones. The properties of the condensation compound are described.

IT IS WELL KNOWN that bis (4-aminophenyl)-sulfone is highly active against a number of organisms, but that the compound is also highly toxic. By condensing bis(4-aminophenyl)-sulfone with L-ascorbic acid, Sah, *et al.*, (1), obtained, with a very high yield, a crystalline product which is comparatively nontoxic, having an LD_{50} of 1.0 to 2.6 Gm./Kg. (oral, mice and rats), and which causes complete inhibition of growth of *Mycobacterium tuberculosis*, H37Rv, at a concentration of 0.2 mg. per 10 cc. of culture. This observation was confirmed by Anderson and co-workers, who also reported the *in vivo* activity of this new drug against murine leprosy (2).

Jones (3) made a careful study of the *in vivo* activity of this new drug in mice infected with *Mycobacterium tuberculosis*, H37Rv, and found the results comparable with those obtained by treating the infected mice with *p*-aminosalicylic acid or *p*-hydroxybenzaldehyde thiosemicarbazone (4). Smith and Chang of the National Institutes of Health, Bethesda, Md. (5) treated guinea pigs infected with *M. tuberculosis*, H37Rv, with this new drug at a daily dosage of 300–500 mg./Kg. for a period of ten weeks. In the final appraisal, the chemotherapeutic effective-

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† Presented to the Medicinal Section, Twelfth International Congress of Pure and Applied Chemistry, New York, September, 1951.

ness (CE) was found to be 3 (6). This figure compares favorably with the chemotherapeutic effectiveness of other well-known sulfone drugs (7); see Table I.

Most recently, Anderson and Contpoulos treated a number of tuberculous patients in the Hamlin Memorial Sanatorium, Hammana, Lebanon, with this new drug in comparison with the mono-2-hydroxyethyl derivative of bis(4-aminophenyl)-sulfone. From the preliminary results (10), it was found that about 50 per cent of the treated patients are improving and that there is no perceptible difference between the two compounds.

TABLE I.—COMPARISON OF CHEMOTHERAPEUTIC EFFECTIVENESS (CE) OF WELL-KNOWN SULFONE DRUGS (9)

Sulfone Drugs	C. E. in TB Animals (TB of control/TB of treated)
Bis(4-aminophenyl)-sulfone (DDS)	2.0
Promin	2.3
Diasone	1.2
Promizole	1.5
Sulfotrone	1.4
Mono-D-galacturonyl derivative of DDS	2.7
Mono-2-hydroxyethyl derivative of DDS (8)	2.5
Di-L-ascorbic acid derivative of DDS	3.0

STRUCTURE AND PROPERTIES

In view of these interesting findings, it is deemed important and necessary to study thoroughly the chemical constitution as well as the characteristic properties of this new drug, the structure of which is not yet established.

There are several possibilities for the structure of the compound formed by condensing bis(4-aminophenyl)-sulfone with L-ascorbic acid. The yield of the compound is almost quantitative when two moles of ascorbic acid react with one mole of the sulfone; hence, it is almost certain that the resulting compound contains two ascorbic acid residues to one of the sulfone. The possible structures are:

A salt (I).

An imine (Schiff base) with the union at the 2-carbon atom in ascorbic acid (II).

An imine (Schiff base) with the union at the 3-carbon atom in ascorbic acid (III).

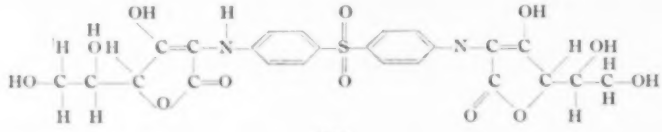
A tautomer of II (IV).

TABLE II

	Observed	Theoretical for $C_{24}H_{24}O_{12}N_2S$	Theoretical Salt $C_{24}H_{28}O_{14}N_2S$
C	50.98 50.92	51.06	48.00
H	4.31 4.33	4.27	4.70
N	5.01 4.92	4.96	4.67
S	5.72 5.66	5.68	5.34

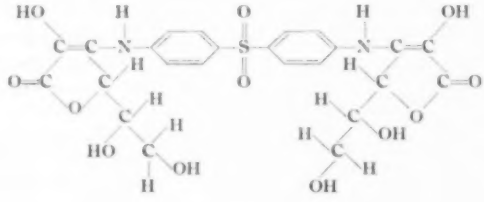
later. On the other hand, the observed values for C, H, N, and S are in very good agreement with the molecular formula $C_{24}H_{24}O_{12}N_2S$, which would result if two molecules of ascorbic acid condensed with one molecule of the sulfone with the elimination of two molecules of water.

The titration curve of the compound in 50% alcohol shown in Fig. 1 indicates two dissociable



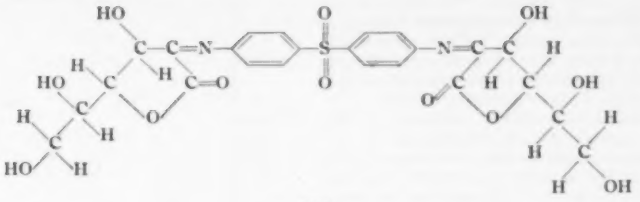
(IV)

(V) A tautomer of III.

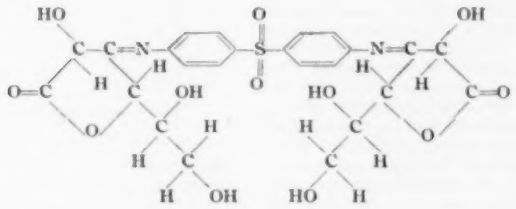


(V)

The analysis of the compound as reported by C. Y. Chiao is shown in Table II. The analysis data show that the compound is not a salt, and this conclusion is further substantiated by evidence reported hydrogen atoms in the molecule. The equivalent weight from this titration was 274, while the molecular weight corresponding to any of the proposed structures II–V would be 564, half of which is 282,



(II)



(III)

a value fairly close to the observed value. The pK_a values for these two hydrogens as taken from the curve, are 4.57 and 5.45, values which are to be compared with the pK_a of 4.85 for L-ascorbic acid in 50% alcohol (11). The second pK_a is smaller than the first, due to the negative charge on the ion.

The comparatively high acidity of the compound rules out the possibility that the compound has either of the two imine structures II or III, since these would have acid strengths considerably weaker than a pK_a of 4.5; the only potentially acidic hydrogens here are from alcoholic hydroxyl groups. Their acidity would be that of an alcohol $K_a \approx 10^{-10}$ augmented by the inductive effect of the negative groups which might bring the K_a's up to 10^{-12} or 10^{-13}.

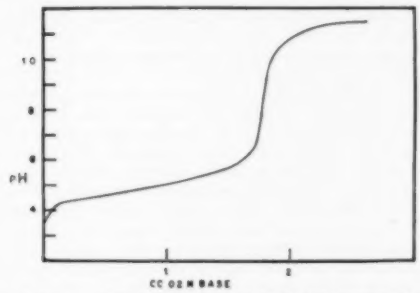


Fig. 1.—Titration curve of bis(4-aminophenyl)-sulfone L-ascorbic acid condensation product in 50% ethanol-water solution.

The high acidity is evidence against structure V also, for this has the stronger acid OH replaced with the sulfone residue. The OH on the 3-carbon atom is known to be much stronger than the OH on the 2-atom.

Structure IV would be expected to have an acid strength comparable to that of ascorbic acid, since the compound has the same type of acid-strengthening resonance as ascorbic acid, and the group replacing the OH on carbon atom 2 has considerable electronegative character. Structure IV, then, can adequately account for the observed acid strength.

The salt structure is ruled out on several counts. The amino groups in the sulfone would have a pK_b of about 12 and this is too weak to form an appreciable amount of salt with ascorbic acid of pK_a 4.12 (in water). The titration curves of such an acid and base indicate that the equilibrium would be against the salt and in favor of the free acid and base.

The compound does not reduce 2,4-dichlorophenylindophenol with any appreciable speed, but such would not be the case if the compound were a salt, since ascorbic acid and its salts reduce this compound very rapidly. The spectrum and the dipole moment of the compound, which are discussed later, also are evidence against the salt structure.

If a water solution of the compound is boiled, however, the resulting solution does reduce 2,4-dichlorophenolindophenol. This apparently results from hydrolysis and indicates that the compound hydrolyzes rather easily. It was also observed that samples of the compound which had stood in bottles for several months also reduced the reagent, presumably because of some hydrolysis from moisture in the air.

Further evidence in regard to whether the attachment is at the 2- or the 3-carbon atom in the ascorbic acid portions of the molecule is given by the properties of the compound compared with those of the 2- and 3-methyl ascorbic acids. 2-Methyl ascorbic acid has reducing properties (12) and does not give a blue color with ferric chloride. 3-Methyl ascorbic acid reduces neither an acidified iodine solution nor Tollens' solution, but gives an intense blue color

with aqueous ferric chloride. The bis(4-aminophenyl)-sulfone ascorbic acid compound reduces an acidified iodine solution and Tollens' reagent and does not give a color with ferric chloride. Clearly the compound behaves like a 2-substituted ascorbic acid and not like a 3-substituted ascorbic acid.

The ultraviolet absorption spectrum of the compound, together with the spectra of its precursors, gives additional evidence with regard to its structure. In Fig. 2, curve *1* shows that the compound has two peaks, one at 254 mμ with a molecular extinction coefficient ε of 27,200 and another at 294 mμ with an ε of 21,800. Curve *2* is the spectrum of L-ascorbic acid multiplied by two, since there are two ascorbic acid residues in the molecule. Curve *4* is the sum of curve *2* plus curve *3*, the spectrum of bis(4-aminophenyl)-sulfone. It is apparent that the 294 mμ band in the compound is due to the 295 mμ band present in the sulfone, while the 254 mμ band arises from a combination of the 246 mμ band in ascorbic acid plus the 260 mμ band present in the sulfone. The two peaks in the compound occur at almost identical wave lengths with the two peaks in curve *4*, which is the sum of the curves for the separate components. The extinction coefficient

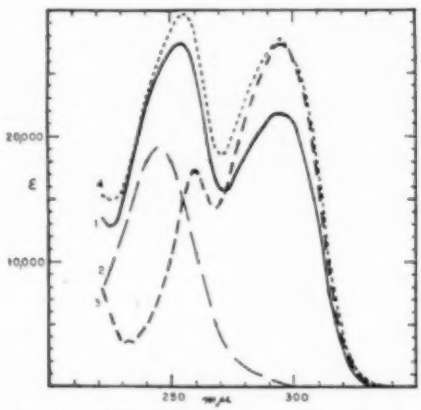


Fig. 2.—Molecular extinction coefficient, ε, in 95% ethanol vs. wave length, λ, in millimicrons: Curve 1 ——, is for the bis(4-aminophenyl)-sulfone ascorbic acid compound; Curve 2 — — is twice the ε value for ascorbic acid; Curve 3 – – – is for bis-(4-aminophenyl)-sulfone. Curve 4 - - - - - is obtained by adding curves 2 and 3.

of the long wave-length band in the compound is considerably lower, however, and the extinction coefficient of the short wave-length band somewhat lower, making it unlikely that we are dealing with a mixture or a simple salt. On the other hand, the spectra represented by curves *1* and *4* are sufficiently alike to indicate that in the compound, a not very profound change has taken place with respect to the resonating structures responsible for light absorption in the original components. Of the structures II–V, structure IV would involve but little disturbance to those original resonating structures. The Schiff base structures II and III no longer have the resonating system in the ascorbic acid portion

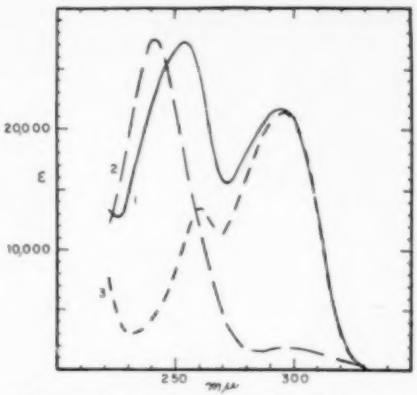


Fig. 3.—Curve 1 ——, molecular extinction coefficient vs. wave length; bis(4-aminophenyl)-sulfone ascorbic acid compound in 95% ethanol. Curve 2 — —, acid added to the above solution. Curve 3 – – –, base added to the above solution.

intact, and the amino-phenyl-sulfone resonance would also be altered considerably, due to the double-bonded nitrogen atom.

Figure 3 shows that upon adding base to the solution, the 254 mμ peak disappears and the spectrum of the compound reverts to that of the sulfone. This may involve the hydrolysis and destruction of the ascorbic acid portion of the molecule. We have evidence that the compound hydrolyzes rapidly in basic solution, and it is well known that ascorbic acid undergoes rapid oxidation in basic solutions. The addition of hydrochloric acid wipes out the 294 mμ peak and also the 260 mμ peak, both of which, as we have shown, come from the aminophenyl-sulfone part of the molecule. The resulting spectrum is very similar to that of ascorbic acid. This is accounted for on the basis that the nitrogen, after accepting a proton, no longer is able to resonate with the phenylsulfone portion of the molecule (13); hence, the compound no longer has much absorption from this part. This explanation is valid whether or not the compound has undergone hydrolysis.

Of the two structures IV and V, the resonance in IV deviates less from the resonance in the individual components. It has been reported that 2-methyl ascorbic acid (12) has a spectrum characteristic of ascorbic acid, while the spectrum of 3-methyl ascorbic acid is somewhat different (14). Also, in structure V the main ascorbic acid resonance would put a plus charge on the nitrogen atom which would then oppose the aminosulfone resonance. Thus a much greater difference between the spectrum of the compound and the sum of the component spectra would be anticipated for V than for IV. Structure IV, then, is the only one of the five that adequately accounts for the ultraviolet spectrum of the compound.

The infrared spectrum of the compound was measured and compared with the spectrum of a mixture of the components. Some bands appear with the compound that are not present in the mixture, agreeing with the previous evidence that the substance is not a mixture.

The optical rotation of the compound was found to be $[\alpha]_D^{23} = +39.4°$ measured in absolute ethanol at a concentration of 0.2 to 0.5%. L-Ascorbic acid under the same conditions had a rotation of $[\alpha]_D^{23} = +57.7°$.

The compound has a solubility in water of 0.4 Gm. per 100 ml. at 30°.

When the compound was refluxed with 0.2 *N* sodium hydroxide, the basic solution soon turned yellow. After heating for three hours and back-titrating, an equivalent weight of 82 was obtained, indicating that the ring structures of the ascorbic acid portions of the molecule were broken up with the production of additional acid groups. Upon standing, the solution deposited white crystals which melted at 175–176° and were identified as bis(4-aminophenyl)-sulfone; m.p. 175–176°.

Upon refluxing with 0.2 *N* hydrochloric acid, a brown precipitate appeared; after three hours the solution was titrated and an equivalent weight of 245 was obtained (not greatly below the value of 274 obtained when the compound was titrated directly). The ascorbic acid portion of the molecule apparently did not break up to release additional acid groups in acid solution; the resin-like precipitate possibly came from the polymerization of a furfural-like compound resulting from the decomposition of the ascorbic acid portion of the molecule in the presence of hydrochloric acid.

The dipole moment of the compound was measured in dioxane solution at 30°. The dipole moment was calculated, using the equation of Halverstadt and Kumler (15).

$$p_{2\infty} = \frac{3av_1}{(\epsilon_1 + 2)^2} + (v_1 + \beta)\frac{(\epsilon_1 - 1)}{(\epsilon_1 + 2)}$$

$$P_{2\infty} = p_{2\infty} M_2$$

$$\mu = 0.0128 \sqrt{(P_{2\infty} - P_{E_2})T}$$

The plot of ϵ_{12} against ω_2 and v_{12} against ω_2 is shown in Fig. 4. The following values were found: $\epsilon_1 = 2.1991$; $v_1 = 0.97386$; $\alpha = 16.46$; $\beta = -0.345$; $P_{2\infty} = 1648$. P_{E_2} was calculated from the atomic refractions to be 125 and the dipole moment was found to be 8.70 D. This moment is consistent with the moment expected for a structure such as IV

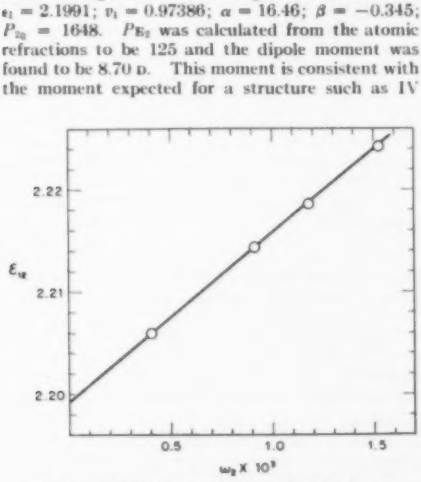


Fig. 4.—Dielectric constant-weight fraction ϵ_{12}-ω_2 plot for bis(4-aminophenyl)-sulfone ascorbic acid compound, bis[4-(2-ascorbamido)phenyl]sulfone.

made up of ascorbic acid and an aminosulfone without a great amount of disturbance to either original structure. Ascorbic acid has a dipole moment of 3.93 D (16) and sulfones and aminophenyl sulfonamides have moments of the order of 5–7 D, so the observed value of the compound is within the expected range. The observed moment is below that expected for a salt.

The molecular weights of the compound and some related compounds, as determined by the boiling point method, are shown in Table III. It is at once apparent that the molecular weight of the compound in both alcohol and dioxane is considerably less than for the molecular formula that agrees with the analysis. The low value in alcohol could be explained on the basis of alcoholysis which (if such takes place with the formation of three particles) would have a theoretical value of 188. The great ease of hydrolysis is indicative that alcoholysis would also take place with ease.

The observed molecular weight in dioxane, however, cannot be explained on such a basis, for dioxane is an ether and would not produce alcoholysis. It is possible in this case that the low value may be due to the compound, which is a relatively strong acid, forming an oxonium salt with dioxane which could give rise to two particles. Dioxane is known to form compounds of this type with certain strong acids; for example, sulfuric acid forms a solid which melts at about 100°.

It is to be noted that while L-ascorbic acid, bis-(4-aminophenyl)-sulfone, and sulfanilamide give observed molecular weights near the theoretical, the compound bis(4-propionyl-aminophenyl)-sulfone also gives a molecular weight less than the theoretical when measured in alcohol. Apparently compounds of this type are also subject to alcoholysis, although to a considerably smaller extent than the ascorbic acid-sulfone compound.

The available evidence indicates that the behavior of the molecule is best accounted for by structure IV, which has two ascorbic acid residues joined at the 2-carbon atom to the two nitrogen atoms of the sulfone with single bonds. There are serious objections to all the other structures. The salt structure is definitely ruled out on several counts; namely analysis, dipole moment, lack of reaction with 2,6-dichlorophenolindophenol, ultraviolet absorption spectrum, and the acid and base dissociation constants of the reactants. The imine structures are ruled out on the basis of the acid dissociation constant and the ultraviolet spectrum of the compound. The structure with the attachment at the 3-carbon atom (structure V) is ruled out on the basis of the lack of reaction with ferric chloride, the reduction of Tollens' solution and acidified iodine solution, the absorption spectrum, and the acid dissociation constant.

On the other hand, the structure with the attachment at the 2-carbon atom (structure IV) accounts very well for the acid dissociation constant, the ultraviolet spectrum, the lack of reaction with ferric chloride, the reaction with Tollens' solution, and the lack of reaction with 2,6-dichlorophenolindophenol. This structure also can account for the observed molecular weights in alcohol and dioxane because of alcoholysis and oxonium salt formation. The name of a compound with this structure would be bis[4-(2-ascorbamido)phenyl]sulfone.

TABLE III.—MOLECULAR WEIGHTS

Compound	Observed Ethanol	Observed Dioxane	Theoretical Salt	Theoretical $C_{18}H_{22}O_{12}N_2S$
Bis(4-aminophenyl)-sulfone	156	272	200.2	564.5 Half 282
L-ascorbic acid	153	273		...
	151	271		...
L-Ascorbic acid	164	...		176
	176	...		...
Bis(4-aminophenyl)sulfone	247	...		248
	252	...		...
Sulfanilamide	...	167		172
	...	172		...
Bis(4-propionylaminophenyl)-sulfone	318	...		360
	330	...		...

SUMMARY

The compound formed by condensing L-ascorbic acid with bis (4-aminophenyl)-sulfone has a pK_a of 4.57 in 50 per cent alcohol solution and a dipole moment of 8.70 D. It does not react with 2,6-dichlorophenolindophenol or with ferric chloride, but it reduces Tollens' solution and acidified iodine solution.

Its ultraviolet absorption spectrum differs, but not markedly, from the additive spectra of the two compounds from which it is made. The elementary analysis corresponds to a compound formed between two molecules of L-ascorbic acid and one of the sulfone with the elimination of two molecules of water. These properties are accounted for by the structure bis [4-(2-ascorbamido) phenyl] sulfone, and are not consistent with any of the other possible structures that were considered.

The observed molecular weight of the compound by the boiling-point method is about one-third the expected molecular weight in alcohol and about one-half the expected molecular weight in dioxane. This is accounted for on the basis of alcoholysis and oxonium ion formation, respectively, in the two solvents.

REFERENCES

(1) Sah, P. P. T., Daniels, T. C., Chen, K. K., and Kleiderer, E. C., *Rec. trav. chim.*, **68**, 118(1949).
(2) Bierwagen, O., Chin, Y. C., Anderson, H., Abstract of the American Society for Pharmacology and Experimental Therapeutics, Fall Meeting, Boston, November, 1950.
(3) Jones, F. G., Progress Report of the Eli Lilly Research Laboratories, 1950.
(4) Sah, P. P. T., and Daniels, T. C., *Rec. trav. chim.*, **69**, 1548(1950).
(5) Smith, M. I., and Chang, Y. T., private communication.
(6) Sah, P. P. T., *Rec. trav. chim.*, **69**, 277(1950).
(7) Smith, M. I., Jackson, E. L., and Bauer, H., *Ann. N. Y. Acad. Sci.*, **52**, 704(1949).
(8) Jackson, E. L., *J. Am. Chem. Soc.*, **70**, 680(1948); **78**, 395(1950).
(9) Smith, M. I., *et al.*, *Am. Rev. Tuber.*, **60**, 62(1949).
(10) Anderson, H. H., private communication.
(11) Kumler, W. D., and Daniels, T. C., *J. Am. Chem. Soc.*, **57**, 1929(1935).
(12) Haworth, W. N., Hirst, E. L., Smith, F., and Wilson, W. J., *J. Chem. Soc.*, **1937**, p. 829.
(13) Kumler, W. D., and Strait, L. A., *J. Am. Chem. Soc.*, **65**, 2349(1943).
(14) Haworth, W. N., and Hirst, E. L., *Helv. Chim. Acta*, **17**, 520(1934).
(15) Halverstadt, I. F., and Kumler, W. D., *J. Am. Chem. Soc.*, **64**, 2988(1942).
(16) Kumler, W. D., *J. Am. Chem. Soc.*, **62**, 3292(1940).

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Ursolic acid
1,12-Dodecanedioic acid
Tyrosol
Tryptophol
Sphingosine
Pyrithiamine
Nicotyrine
Mellitic acid
Lignoceryl alcohol
sym.-Diisopropyl hydrazine
dl-Ethionine
Hexokinase
Lumiflavin
Cerotic acid
l-Camphor
Ascaridol
Coronene
1,9-Benzanthrone
Iodol

Iodine Solution as a Sporicidal Agent*,†

By LOUIS GERSHENFELD and BERNARD WITLIN

Since the latter part of the 19th century, much has been written extolling the alleged virtues of iodine as an antiseptic. The sporicidal action of iodine has been reported but discrepancies appear in the results. Comparative studies have been made between water and water-alcoholic iodine solutions in which the water solutions have consistently been shown to be more efficient. This *in vitro* study reports on the sporicidal efficiency of a 2 per cent water-iodine solution (iodine solution N. F. IX). Both "wet" and "dry" techniques were employed, using conditions with different *p*H values. Iodine solution N. F. IX was found to be effective against the spores of *B. anthracis, B. subtilis, B. megatherium, B. mesentericus,* and *Cl. tetani.*

THE SPORICIDAL ACTION of iodine has been reported and known since 1873 (1). Data concerning the efficiency of iodine against the spores of *B. anthracis* (2–4), *Cl. tetani* (3, 6, 7), and *Cl. welchii* (3, 4), both *in vitro* and *in vivo* findings, have been noted. However, there have been discrepancies (5) in the results reported. This may be due to the variations in the techniques used in testing (5) and, in part, to the free iodine content of the preparations under test.

Since the latter part of the 19th century, much has been written extolling the alleged virtues of iodine as an antiseptic. Preparations containing free iodine used as antiseptics are usually prepared today by dissolving iodine with soluble iodides in water or diluted alcohol. Comparative studies have been made between aqueous and hydroalcoholic iodine solutions in which the aqueous solutions have consistently been shown to be more efficient (7–10).

This *in vitro* study was undertaken to determine the sporicidal efficiency of a 2 per cent aqueous iodine solution (iodine solution N. F. IX).

EXPERIMENTAL

General Procedure.—Spores of *B. anthracis, B. subtilis, B. megatherium. B. mesentericus,* and *Cl. tetani* were employed. The procedure consisted of saturating sterile 1-cm. squares of Whatman No. 1 filter paper separately with saline suspensions of heat-treated (70° for thirty minutes), one-year old cultures (spores). At room temperature (22–27°), the spores in each instance were washed off agar slants with isotonic saline and standardized so that the quantity of suspension (0.01 ml.) absorbed by each 1-cm. square of filter paper contained approximately 500,000 spores of each species. The sterile filter-paper squares were dipped in the individual spore suspensions, the excess suspension was drained, and the squares were placed in sterile Petri dishes covered with Brewer "open type" covers. The squares were then placed in the incubator at 37° and incubated to dryness (forty-eight hours).

The dried spore-impregnated filter-paper squares were transferred to sterile Petri dishes and covered with 10 ml. of iodine solution, N. F. At thirty-minute intervals, for a period of six hours, the squares were removed with sterile forceps, rinsed in one 10-ml. tube of sterile broth[1], transplanted to another tube of 10 ml. of broth, and incubated for seven days at 37°.

Another series was treated as above, employing, however, a sterile isotonic saline rinse, followed by immersion in sterile, 1% sodium thiosulfate (until the disappearance of the iodine color), and then by another rinse in sterile isotonic saline (to remove the thiosulfate) prior to transplanting into broth.

Findings.—In *in vitro* tests, iodine solution N. F. (2%) was capable of destroying the spores of *B. subtilis, B. anthracis,* and *B. mesentericus* within two and one-half hours and *Cl. tetani* within one and one-half hours. Under conditions of this test, spores of *B. megatherium* were not destroyed after an exposure of five and one-half hours.

In vivo tests, using *Cl. tetani* and other pathogenic spore-bearers, are being conducted.

Increasing the Number of Spores.—To note the effect iodine solution N. F. would have on an increased number of spores, the first procedure was repeated, using 1,000,000 spores per ml. instead of 500,000 per ml.

Findings.—The sporicidal efficiency of iodine solution N. F. IX was the same for 1,000,000 spores per ml. as for 500,000 per ml.

***p*H Variation.**—The general procedure, using 1,000,000 spores per ml., was repeated with the test organisms against iodine solution N. F. adjusted immediately before testing with hydrochloric acid to *p*H values of 1.0, 2.0, 3.0, and 4.0. Hydrogen-ion concentration measurements were made with a Beckman electric potentiometer (Model H). Controls of hydrochloric acid in distilled water, adjusted to the desired *p*H values, were run simultaneously with controls of iodine solution N. F.

Findings.—Iodine solution N. F. IX possessing a *p*H value of 4.6 was capable of destroying the spores of *B. subtilis, B. anthracis,* and *B. mesentericus* within two and one-half hours and *Cl. tetani* within one and one-half hours. The spores of *B. mega-*

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† This investigation was conducted at the Philadelphia College of Pharmacy and Science, Department of Bacteriology, under a grant from the Chilean Iodine Educational Bureau Fellowship, Mellon Institute.

[1] With the exception of *Cl. tetani,* F. D. A. broth was employed for all experiments. For *Cl. tetani,* thioglycollate medium (steamed and cooled prior to inoculation) was used throughout.

therium were not destroyed after an exposure of five and one-half hours. Controls of hydrochloric acid in water at *p*H 4.6 were ineffective against all of the test spores within five and one-half hours.

Iodine solution N. F. IX adjusted to *p*H 4.0 with hydrochloric acid destroyed the spores of *B. subtilis*, *B anthracis*, *B. mesentericus*, *B. megatherium*, and *Cl. tetani* within ninety minutes. Controls of hydrochloric acid in water at *p*H 4.0 were ineffective against all of the test spores within five and one-half hours.

Iodine solution N. F. IX adjusted to *p*H 3.0, 2.0, and 1.0 with hydrochloric acid was capable of destroying the spores of *B. subtilis*, *B. anthracis*, *B. mesentericus*, *B. megatherium*, and *Cl. tetani* within sixty minutes but not in thirty minutes. Hydrochloric acid controls were ineffective within five and one-half hours.

Sporicidal Testing on Nonabsorbable Surface (Wet).—Injector-type razor blades (Schick[3]) were washed in acetone to remove any adhering oil or grease and then were sterilized in Petri dishes in the hot-air oven at 170° for two hours. The blades were dipped, by means of a platinum hook, into the spore suspensions of 1,000,000 per ml., allowed to drain for one minute, and then placed individually in 15 ml. of iodine solution N. F. IX contained in a seeding tube (85 x 25 mm.) At designated intervals of time, the blades were removed, drained of excess iodine, immersed in sterile 1% sodium thiosulfate solution, rinsed in sterile isotonic saline, and then transplanted into 10 ml. of broth. The transplants were incubated at 37° for seventy-two hours and then were observed for the presence or absence of growth.

Findings.—The times required for iodine solution N. F. IX to kill the spore suspensions adhering to the razor blades were as follows: *B. anthracis*, ninety minutes; *B. subtilis*, ninety minutes; *B. mesentericus*, ninety minutes; *B. megatherium*, four hours; and *Cl. tetani*, ninety minutes.

Sporicidal Testing on Nonabsorbable Surface (Dry).—The procedure using the razor blades was followed except that the blades, after being dipped in the spore suspensions, were allowed to dry for seventy-two hours at 37° by placing them in sterile Petri dishes with Brewer open-type covers. The dry blades were then immersed individually in 15 ml. of iodine solution N. F. IX in seeding tubes. At designated intervals of time, the blades were removed, drained of excess iodine, immersed in sterile 1% sodium thiosulfate, rinsed in sterile isotonic saline, and transplanted into 10 ml. of broth. The transplants were incubated at 37° for seventy-two hours and then were observed for the presence or absence of growth.

[3] Manufactured by Eversharp, Inc., New York, N. Y.

Findings.—The times required for iodine solution N. F. IX to kill the spore suspensions dried onto the razor blades were as follows: *B. anthracis*, five hours; *B. subtilis*, three hours; *B. mesentericus*, four hours; and *Cl. tetani*, two hours. *B. megatherium* spores were not destroyed within five and one-half hours of exposure.

SUMMARY AND CONCLUSIONS

An *in vitro* study was made to determine the effect of iodine solution N. F. IX (*p*H 4.6) upon one-year-old spores of *B. anthracis*, *B. subtilis*, *B. megatherium*, *B. mesentericus*, and *Cl. tetani* by both "wet" and "dry" techniques. The same procedures were used employing lower *p*H values (4.0, 3.0, 2.0, and 1.0).

The results observed in this series of experiments revealed that:

1. Two per cent aqueous iodine (iodine solution N. F. IX) possesses sporicidal efficiency.

2 The efficiency of iodine solution N. F. IX against the spores of *B. subtilis*, *B. anthracis*, *B. mesentericus*, *B. megatherium*, and *Cl. tetani* was enhanced at lower *p*H values (4.0, 3.0, 2.0, and 1.0). Controls of hydrochloric acid in water at the same *p*H values were ineffective against all of the test spores under the conditions of the procedures used in this study.

3. Wet suspensions of spores on an absorbable surface (filter paper) required longer periods of exposure than those on nonabsorbable surfaces (razor blades) before being killed.

4. Spores dried on an absorbable surface (filter paper) required shorter periods of exposure than those on nonabsorbable surfaces (razor blades) before being killed.

REFERENCES

(1) Davaine, "Dictionaire Encyclopedie des Sciences Med.", P. Asselin and G. Masson, Paris, 1873, p. 335.
(2) Claudius, *Deut. Z. Chirurgie*, **44**, 489(1902).
(3) Tinker, M. E., and Sutton, H. B., *J. Am. Med. Assoc.*, **87**, 1347(1926).
(4) Simmons, J. S., *ibid.*, **91**, 704(1928).
(5) Gershenfeld, L., and Witlin, B., *Ann. N. Y. Acad. Sci.*, **53**, 172(1950).
(6) Gershenfeld, L., and Miller, R. E., THIS JOURNAL, **21**, 894(1932).
(7) Moll, T., *Zentr. Bakt. Parasitenk. Abt. I Ref.*, **6**, 416 (1920).
(8) Beal, G. D., Waters, K. L., and Black, P., THIS JOURNAL, **8**, 354(1947).
(9) Gershenfeld, L., and Witlin, B., *ibid.*, **39**, 489(1950).
(10) Ficarra, B. J., *J. Intern. Coll. Surgeons*, **16**, 115 (1951).

The Pharmacological Activity of Substances Extracted from Spanish Moss, *Tillandsia usneoides* L.*,†

By SELDON DICK FEURT‡ and LAURETTA E. FOX§

Extracts of *Tillandsia usneoides* L., and other species of *Tillandsia*, family *Bromeliaceae*, possess estrogenic activity as tested by several methods. The amount of estrogenic substances in *Tillandsia* exceeds that in many species of plants.

TILLANDSIA USNEOIDES L., family *Bromeliaceae*, commonly called Spanish moss, is an epiphyte which festoons the trees in swamps and hammocks from Virginia to Florida and west to Texas, also southward throughout northern South America. The plant is used for upholstery, for mulching, and occasionally for fodder.

The Florida Experiment Station found that Spanish moss has more food value than oat straw (1), and French and Abbott (2) reported 1,500 μg. of carotene per 100 Gm. of moss. Schorger (3) extracted carbohydrates and waxes from the moss.

Dean (4) and Metzger (5) showed that Spanish moss possessed antigenic properties. Kondrosheff (6) and Mayo and Wakefield (7) used sterilized moss as a substitute for cotton in surgical dressings. The moss will absorb six to ten times its dry weight of water, while cotton will absorb only four to six times its weight.

EXPERIMENTAL

Nature of Extracts.—The fresh green Spanish moss, *Tillandsia usneoides* L., was cut in pieces approximately one-fourth inch long, macerated with water, and extracted with organic solvents, such as benzene, acetone, or chloroform. One hundred grams of Spanish moss yielded 2.5 Gm. of crude extract or 0.20 Gm. of purified extract, which were used in the tests described below. The activity of the extracts varied with the season of the year in which the moss was collected and with the stage of growth of the moss.

Survival Time After Adrenalectomy.—Male albino rats, approximately twenty-one days old, were bilaterally adrenalectomized under pentobarbital sodium anesthesia using a bilateral dorsal approach with a single dorsal skin incision. Immediately after the operation, two milligrams of the crude extract was administered intramuscularly. The survival time of the adrenalectomized rats receiving no extract was from forty-eight to one hundred and ninety-two hours, with an average of one hundred and two hours. The survival time of the adrenalectomized rats receiving the extract was from twelve to eighteen hours, with an average of thirteen and one-half hours. This extract did not prolong the life of adrenalectomized rats, nor did it maintain body weight.

Vitamin D Activity (8).—Albino rats, twenty-seven to thirty days old, weighing approximately 60 Gm., were fed a rachitogenic diet for three weeks. For six days they received orally a daily dose of from 0.5 to 4.0 mg. of the crude extract dissolved in oil. The animals were sacrificed and the tibias sectioned and stained with silver nitrate. There was no evidence of calcification; the proximal ends of the tibias showed involutional changes which were more severe with the higher doses of the extract.

Androgenic Activity by the Chick Comb Method of Hollander (9).—Three groups of eight single-combed white leghorn chicks, sexed and one day old, received respectively, no medication, twenty γ of testosterone propionate in oil daily by topical application, and ten γ daily of the purified extract of Spanish moss in oil by topical application. The experimental chicks received nine applications of the test substances. The chicks were sacrificed on the tenth day, and the body and comb weights determined. The calculations showed no androgenic activity in the Spanish moss extracts. These female chicks showed no depression of body or comb weight.

Weight of Seminal Vesicles of Castrates.—Young male albino rats were castrated and divided into two groups, one of which received 1 mg. of the crude extract of Spanish moss daily for ten days; the other served as controls. The animals were sacrificed on the eleventh day and the weight of the seminal vesicles determined. The average weight of the seminal vesicles of the control group was 23.5 mg., and that of the treated group was 34.6 mg.

Estrogenic Activity as Determined by the Allen-Doisy Vaginal Smear Method (10).—Adult female rats were ovariectomized, and vaginal smears taken periodically for ten days to determine that the rats were not capable of an estrus cycle. The rats received 1 mg. of the crude extract, and a second injection of the same dose twenty-four hours later. Forty-eight hours after the first injection the vaginal smear was characterized by many leukocytes and a few nucleated epithelial cells. At seventy-two hours there were no leukocytes and many nucleated epithelial cells. After ninety-six hours there were many large nucleated squamous epithelial cells. These characteristic cells were indicative of an estrogenic substance in Spanish moss extracts.

Lauson Uterine Weight Assay (11).—Immature female albino rats within the weight range of 34–39 Gm. were divided into control and experimental groups. Each experimental group received 0.01 mg. of the purified extract per 30 Gm. of body weight daily. At the end of the third day both groups were

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† Abstracted in part from a thesis submitted to the Graduate School of the University of Florida by Seldon Dick Feurt in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
‡ Instructor in Pharmacy, College of Pharmacy, University of Florida.
§ Assistant Professor of Pharmacognosy and Pharmacology, College of Pharmacy, University of Florida.

sacrified and the uteri removed by cutting through the cervex and the uterotubal junction. There was only a slight increase in uterine weight of the injected animals.

It was thought that a larger dose of the extract injected over a longer period of time might produce a more marked effect. Immature female albino rats weighing 34–39 Gm. were divided into two groups. One group received 0.1 mg. of the crude extract intraperitoneally daily for six days. The animals were sacrificed on the sixth day, and the uteri removed and weighed. The experimental group with an average initial body weight of 37.36 Gm. and a final body weight of 45.80 Gm. had an average uterine weight of 17.0 mg. or 37.1 mg. % of body weight. The control group, with an initial average body weight of 37.75 Gm. and a final average body weight of 57.30 Gm., had an average uterine weight of 20.5 mg. or 34.0 mg. %. This modified Lauson assay, as well as the Lauson assay, gave a uterine weight curve with a low plateau.

Vaginal Opening in Immature Rats.—Eighteen immature female albino rats of approximately the same age and weight were divided into two groups; one serving as control, and the other receiving 20 γ of the purified moss extract twice a week. After twenty days all of the treated animals, but only two control animals, had complete vaginal opening.

Inhibition of Growth.—The growth of young animals was inhibited by injecting or feeding the extracts of Spanish moss. The weight of one group of immature animals under prolonged treatment was depressed 67%. All immature rats injected or fed the moss extracts showed depression of growth. Mature rats receiving a diet consisting of equal parts of ground Spanish moss and commercial ration showed little variation in growth from those receiving the commercial ration. Fewer young were born to those receiving Spanish moss in the ration and the young produced were dwarf, averaging in one litter 6.5 Gm. at birth as compared to 19.5 Gm. at birth for the offspring of a mother receiving the commercial ration. The dwarf rats perished within fourteen days.

Screening Tests on Other Species of *Tillandsia*.—*Tillandsia tenuifolia* L., *T. juncea* P., *T. balbisiana* S., *T. aloifolia* H., *T. simulata* S., *T. fasciculata* S., and *T. circinata* S. were collected and identified by Mr. Richard Lewis, a student in the College of Agriculture, and Miss Lillian Arnold of the Botany Department of the Experiment Station. These species were extracted by the method described above. The extracts produced the characteristic blue fluorescence under ultraviolet light and induced estrus in ovariectomized rats.

DISCUSSION

The extracts of *Tillandsia usneoides* L. did not maintain body weight nor prolong the life of adrenalectomized rats. Every rat receiving the extracts died in a shorter period of time than those receiving no extract. Estradiol benzoate is toxic to adrenalectomized rats (12).

Extracts of Spanish moss administered orally did not promote calcification of rachitogenic animals. Simpson, *et al.* (13), showed that estrin in one week causes an involution of the proximal growth cartilage of the tibia of young rats.

Moss extract had no effect on comb growth or body weight of sexed white leghorn chicks. Estrogens have no effect on the body weight of pullet chicks. The diol-esters are more efficient than either estradiol or estrone in depressing comb growth of male chicks, but combs of female chicks are not affected (14).

Extracts of Spanish moss caused an increase in weight of the seminal vesicles of castrated rats. Treatment of castrated male rats with estrogens causes a slight weight increase in prostrate and a marked weight increase in seminal vesicles (15).

By testing serial dilutions of the extracts in ovariectomized rats, it was determined that 10 γ of the purified extract produced estrus in ovariectomized rats.

The uterine weight increased only slightly after administration of the extracts of Spanish moss. The uterine weight response to estradiol and estrone is consistent in that 1 γ of estradiol is equivalent to 20 γ of estrone. However, the uterine weight response of estriol is incomplete in that the uterine-weight curve plateaus at a much lower level than in the case of the other two estrogens (16).

The vaginal opening of immature rats further confirms the estrogenic action of the extracts.

Injection or feeding of the extracts inhibited growth. The young rats born to mothers receiving estrogens perish, due to suppression of lactation, loss of maternal instinct, or neglect of mother (17). Dwarf rats result from estrogen administration begun at four to six weeks and continued for several months, but injection begun after full growth is reached does not lead to a marked loss of weight (18). Zondek (19) showed a direct relationship between the inhibition of growth and the quantity of hormone administered. This inhibition affects the skeleton as well as the organs. The action is explained by the inhibiting action of the hormone on the anterior pituitary.

SUMMARY

Tillandsia usneoides L. and other species of the genus *Tillandsia* manifest estrogenic activity when tested by the several methods listed.

REFERENCES

(1) Pickell, J. M., "Use of Moss as Foodstuff," Florida Experiment Station Bulletin II, 1890.
(2) French, R. B., and Abbott, O. D., "Carotene," Florida Agricultural Experiment Station Technical Bulletin No. 444, 1948.
(3) Schorger, A. W., *J. Ind. Eng. Chem.*, **19**, 409(1927).
(4) Dean, G. A., *J. Allergy*, **14**, 340(1943).
(5) Metzger, F. C., *J. Florida Med. Assoc.*, **24**, 99(1943).
(6) Kondrosheff, I. S., *Vrachebnae Delo*, **14**, 1097(1931).
(7) Mayo, C. W., and Wakefield, E. G., *Proc. Staff Meetings Mayo Clinic*, **19**, 65(1944).
(8) "United States Pharmacopeia," Thirteenth Revision, Mack Publishing Co., Easton, Pa., 1947, p. 723.
(9) Comroe, J. H., "Methods in Medical Research," Vol. 2, Year Book Publishers, Inc., Chicago, 1950, p. 322.
(10) Allen, E., and Doisy, E. O., *J. Am. Med. Assoc.*, **81**, 819(1923).
(11) Comroe, J. H., "Methods in Medical Research," Vol. 2, The Year Book Publishers, Inc., Chicago, 1950, p. 278.
(12) Pfiffner, G. A., and Hooker, C. W., *Am. J. Physiol.*, **131**, 441(1940).
(13) Simpson, M. W., Kirbrick, E. A., Becks, H., and Evans, H. M., *J. Endocrinol.*, **3**, 286(1942).
(14) Manso, S. S., and Kosin, I. L., *Endocrinology*, **27**, 687(1940).
(15) Greene, R., and Thompson, D., *ibid.*, **30**, 85(1942).
(16) Comroe, J. H., "Methods in Medical Research," Vol. 2, The Year Book Publishers, Inc., Chicago, 1950, p. 279.
(17) Weichert, C. K., and Kerrigan, S., *J. Endocrinol.*, **5**, 741(1942).
(18) Pincus, G., and Thimann, K. V., "The Hormones," Vol. 2, Academic Press, Inc., New York, 1950, p. 22.
(19) Zondek, B., *Lancet*, **10**, 10(1936).

Note

A Note on Some Salts of Quinacrine*

By EDGAR A. STECK, M. E. AUERBACH, and WERNER BOEHME†

THE widely used antimalarial quinacrine is commonly dispensed as the dihydrochloride, a salt which is somewhat limited in solubility. A number of readily prepared salts of the base, 6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine, have now been examined for solubility and also for resistance to hydrolysis.

Table I compares the properties of the dihydrochloride with those of the newer salts. It will be noted that the disulfamate (1) compares well with the dihydrochloride in stability, and is considerably more soluble. The di(methanesulfonate) of quinacrine (2), which has been used for injection purposes, is even more soluble but is somewhat less stable. Since this work was completed, a Russian patent has been issued on the dilactate (3). The antibacterial activity of these salts has been reported (4, 5). The highly insoluble methylene 1,1′-bis-(2-hydroxy-3-naphthoate) of quinacrine was made to investigate the possible value of slow release of the antimalarial agent.

EXPERIMENTAL

Quinacrine Salts.—Quinacrine methylene bis-1,1′-(2-hydroxy-3-naphthoate) was made by combination of aqueous solutions of commercial quinacrine dihydrochloride with an equivalent amount of the sodium methylene bis-1,1′-(2-hydroxy-3-naphthoate). The precipitated salt was washed with water, alcohol, and acetone; then dried at 100°.

All other quinacrine salts were prepared from quinacrine base, which was isolated as the monohydrate (m. p. 82.4 to 83.8°) of 99.3% purity (assay method: reference 6) from a commercial sample of the dihydrochloride. A solution of the base in benzene was dried by co-distillation of the moisture, and the requisite amount of appropriate acid added in benzene (or benzene-acetone) solution. The yellow salts were dried *in vacuo*.

TABLE I.—SALTS OF QUINACRINE

Salt	Yield, %	M. P., °C.[a]	Solubility[b]	pH 2% Soln.	Hydrolysis[c]	Analyses % Base Calcd.	Analyses % Base Found[d]
Dihydrochloride		253–256	4.2	4.50	13.1	84.4	84.4
Disulfamate	99	151–153	33.0	3.95	12.2	67.3	67.1
Di(methanesulfonate)[e]		136–138[f]	59.3	4.60	14.9	67.5	66.8
Diformate	80	88–93	45.8	4.00	13.2	81.3	81.6
Dilactate[g]	82	115–118	61.4	6.30	27.6	68.9	69.0
Tartrate	85	139–141	9.6	4.50	17.3	72.7	72.4
Citrate[h]	89	130–135	33.1	5.00	21.6	67.6	67.4
Di(acetylsalicylate)	90.5	137–140	7.6	5.85	14.0	52.6	52.4
Methylene 1,1′-bis-(2-hydroxy-3-naphthoate)[i]	96	>300	>0.1			48.5	48.7

[a] Corrected melting point. [b] Solubility in water at 25°, w/v%. [c] Per cent hydrolysis of 0.5% w/v solution after 2 hr. at 100°. This was determined by refluxing 0.5 Gm. of salt in 100 cc. of water for exactly 2 hr., then cooling, and collecting the highly insoluble 6-chloro-2-methoxyacridone. The latter was dried at 100°, and weighed. [d] Assay by dichromate method (reference 6). [e] This salt has been widely known as the (di) musonate. [f] Goissedet and Depois (reference 2) give m. p. 125°. [g] The properties of this salt were not given in the abstract available (reference 3). [h] Somewhat hygroscopic. [i] Dihydrate.

* Received March 1, 1952, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

† Present address: National Drug Company, Philadelphia, Pa.

REFERENCES

(1) Auerbach, M. E., to Winthrop Chemical Co., U. S. pat. 2,383,458; British pat. 594,293.
(2) Goissedet, P. E. C., and Depois, R. L., U. S. pat. 2,092,114.
(3) Magidson, O. Yu., and Grigorovskii, A. N., Russian pat. 53,030 [*Chem. Abstr.*, **34**, 5252 (1940)].
(4) Goetchius, G. R., and Lawrence, C. A., *J. Lab. Clin. Med.*, **29**, 134(1944).
(5) Goetchius, G., and Lawrence, C., *ibid.*, **30**, 145(1945).
(6) Auerbach, M. E., THIS JOURNAL, **26**, 231(1937).

Book Reviews

The Essential Oils. Vol. VI. By ERNEST GUENTHER. D. Van Nostrand Company, Inc., New York, 1952. xvi + 481 pp. Illustrated. 15.5 x 23.5 cm. Price $9.75.

This volume completes the series on the essential oils, previous volumes of which have been reviewed in THIS JOURNAL. The sixth volume is composed of monographs describing individual oils; the arrangement is the same as for the third, fourth, and fifth volumes of the series. In this final volume, essential oils from the following plant families are covered: Ericaceae, Betulaceae, Valerianaceae, Verbenaceae, Cistaceae, Cruciferae, Liliaceae, Iridaceae, Araceae, Palmae, Cyperaceae, Moraceae, Aristolochiaceae, Chenopodiaceae, Ranunculaceae, Euphorbiaceae, Malvaceae, Usneaceae, Podocarpaceae, Pinaceae, Taxodiaceae, and Cupressaceae

The completion of *The Essential Oils* has required many years of field investigations in nearly all of the oil-producing regions of the world, in addition to ten years of literature search, editing, and writing. The task has been complicated by having been carried out during a period of rapid progress in chemistry and involved economic changes, but the final accomplishment has certainly been well worth the effort expended. The editor and those who have contributed are to be congratulated upon the successful completion of a well-conceived project designed to furnish complete information on the essential oils. The value of the work is enhanced by an index to Volumes I–VI inclusive, and the inclusion in the final volume of a botanical classification of essential oil plants.

A Textbook of Pharmacology. By WILLIAM T. SALTER, M.D. W. B. Saunders Company, Philadelphia, 1952. xiii + 1240 pp. Illustrated. 18 x 26 cm. Price $15.

The author of this book, who is professor of pharmacology in the Yale University School of Medicine, has written a unique book which goes quite beyond the scope of most textbooks of pharmacology. The book is written in an informal, chatty style which is typical of the author, but which does not detract in the slightest from the quality of the information presented. It is obviously designed for students who expect to become physicians and as a reference book for medical practitioners. The volume is divided into two parts, nine sections, and fifty-five chapters. Part I discusses the general principles of pharmacology; Part II, drug action on physiological mechanisms; Part III, the application of drugs in clinical medicine; and Part IV, toxicology. In general, each chapter in Part II follows the same pattern, beginning with a historical introduction which is followed successively by a treatment of the nature and source, the chemistry, the pharmacodynamics, the effects on special systems and organisms, toxicity, absorption, metabolism and fate, clinical utilization, and preparations applicable to the type of drug action being discussed.

The book is so broad in scope and so replete with information relating to the many ramifications of pharmacology that it will be found useful not only by medical students as a textbook, but also by pharmacy students for supplementary reading and by pharmacists as a reference book. The volume is documented throughout with pertinent references to the original literature and is provided with an adequate index which facilitates the location of specific information.

Chemical Trade Names and Commercial Synonyms. By WILLIAM HAYNES. D. Van Nostrand Co., Inc., New York, 1951. iv + 279 pp. 16 x 24 cm. Price $5.50.

This is a useful book for anyone who has occasion to identify special names used in the chemical industry. The book presents 20,000 entries arranged in alphabetical sequence and includes many trade names and coded names of chemicals used in the textile, paint and varnish, perfume and cosmetic, agricultural, and many other industrial fields. No attempt has been made, however, to include generic and trade-mark names of chemicals used as drugs, although the book does list names of many intermediates used in the pharmaceutical industry. For that reason it should be useful to chemists and purchasing agents in the pharmaceutical industry. Throughout the book, many manufacturing sources are indicated, and a key to manufacturers listed with their addresses constitutes the first twenty-two pages of the book. Mr. Haynes' listing of chemical trade names and commercial synonyms should fill a useful purpose.

The Art of Eloquence. By THEODORE R. MCKELDIN and JOHN C. KRANTZ, Jr. The Williams and Wilkins Company, Baltimore, 1952. x + 245 pp. 15 x 22.5 cm. Price $3.

Ordinarily only books dealing with the basic sciences of pharmaceutical interest are reviewed in these columns. An exception has been made in the case of this book, because one of the authors, Dr. John C. Krantz, Jr., is so well known in pharmaceutical circles. Both authors are noted for their accomplishments in fields other than public speaking, and the appearance of this volume emphasizes the versatility of each.

About half the book is devoted to hints on how to plan and deliver speeches in an effective manner. In this section, such important subjects as vocabulary building, voice modulation, the technique of speaking, and many other problems connected with speaking in public, are adequately treated. In the second portion of the book are reproduced speeches of different types which have been used successfully by the authors. The book can be recommended to anyone who wishes to improve his performance in public speaking, and the authors are to be congratulated upon the success of their undertaking.

Chemical Calculations. By SIDNEY W. BENSON. John Wiley & Sons, Inc., New York, 1952. xi + 217 pp. 15 × 23 cm. Price $2.95.

Mathematics is the language the scientist uses to express the results of his research. Often the student of the laboratory sciences is discouraged early in the game by his inability to do the mathematical calculations he encounters in his studies.

In *Chemical Calculations*, Sidney W. Benson offers a system, in compact form, designed to aid the beginner in his mastery of the mathematics of chemistry. The first chapter presents a clear, step-by-step method of analyzing and setting up any problem, which, if carefully read and followed, should eliminate much of the basic difficulty. Succeeding chapters cover broad topics (such as "The Properties of Gases," "Chemical Equilibrium," "Oxidation and Reduction," etc.) and are further subdivided into brief discussions of the principles involved, with several typical examples shown and worked out. Additional problems are given at the end of each chapter, and there are three useful appendices—a brief summary of the most important mathematical definitions and operations; a table of common units; and, of course, the answers to the problems at the ends of the chapters. The book is carefully indexed; convenient tables of atomic weights and of logarithms are included.

Possibly the only question raised might concern the scope of the volume; the author states that the book is intended specifically for the first-year student, yet a fair amount of space is given to types of calculations which most first-year courses do not stress or go into at length. However, such material would undoubtedly prove valuable for later reference. Nearly all of the subject matter is very much to the point, and *Chemical Calculations* could well serve as an adjunct to the first year's study of chemistry; it might conveniently be integrated with the laboratory course.

Methods and Reactions of Organic Analysis. Volume I. *General Methods of Analysis.* By MAURICE PESEQ and PIERRE POIRIER. Masson & Company, Paris. 276 pp. 17 x 25.5 cm. Price 1,800 fr.

This volume, the first of three, was prepared by the authors with the collaboration of R. Berrett, J. Methieu, and A. Petit to correlate the currently accepted techniques in the field of organic analysis.

The first section of the book is devoted to brief discussions of physical constants and characteristics, and the methods by which they are determined. This is followed by a very brief section on the methods used to identify the elementary components of organic material.

Sections III and IV give procedures for quantitative elementary analysis and for the determination of functional groups. The basic methods are discussed, and specific procedures are given in detail. Where special apparatus is required, diagrams or photographs are included.

Section V reviews several methods for moisture determination, including the method of Karl Fischer.

The appendix contains information about solvents, including methods for purification. It also includes extensive tables of color and fluorescent *p*H indicators. Formulas for mixed indicators are presented, and the use of dithizone as an indicator is mentioned.

The references that are given as footnotes throughout the text are collected in an alphabetical listing of the authors cited. A separate list of references, arranged numerically, is furnished for the section on indicators. A complete table of contents is given, but no subject index is included.

The book is a very good reference and review text. The presentation is brief, but few words are wasted. Volume II of this series will be devoted to methods of identification.

☆

BOOKS RECEIVED

Chemical Trade Names and Commercial Synonyms. By WILLIAM HAYNES. D. Van Nostrand Co., Inc., New York, 1951. iv + 279 pp. 16 x 24 cm. Price $5.50.

Probit Analysis. 2nd ed. By D. J. FINNEY. Cambridge University Press, New York, 1952. 22.5 x 14 cm. xiv + 318 pp.

The Essential Oils. Vol. VI. By ERNEST GUENTHER. D. Van Nostrand Co., Inc., New York, 1952. xvi + 481 pp. Illustrated. 15.5 x 23.5 cm. Price $9.75.

Experimental Nucleonics. By ERNST BLEULER and GEORGE J. GOLDSMITH. Rinehart & Co., Inc., New York, 1952. xv + 393 pp. 16 x 23.5 cm. Price $6.50.

A Textbook of Pharmacology. By WILLIAM T. SALTER, M. D. W. B. Saunders Co., Philadelphia, 1952. xiii + 1240 pp. Illustrated. 18 x 26 cm. Price $15.

Review of Pharmacy. 7th ed. By GEORGE W. FIERO. John Wiley & Sons, Inc., New York, 1952. 122 pp. 16 x 23.5 cm. Price $3.25.

General Chemistry. 6th ed. By HORACE G. DEMING. John Wiley & Sons, Inc., New York, 1952. xii + 656 pp. 15.5 x 23.5 cm. Price $5.75.

Heterocyclic Compounds. Vol. 3. Edited by ROBERT C. ELDERFIELD. John Wiley & Sons, Inc., New York, 1952. vi + 442 pp. 15.5 x 23.5 cm. Price $12.

Heterocyclic Compounds. Vol. 4. Edited by ROBERT C. ELDERFIELD. John Wiley & Sons, Inc., New York, 1952. vi + 674 pp. 15.5 x 23.5 cm. Price $17.

Interim Revision Announcement

PHARMACOPEIA OF THE UNITED STATES, FOURTEENTH REVISION

Four revisions of the U. S. P. XIV have been approved, effective from June 15, 1952:

BENZOIN

Benzoin, page 78—In the paragraph on *Unground Sumatra Benzoin* replace the sentence, "Its odor is aromatic." with the following:

Its odor is aromatic and balsamic. When heated it does not emit a pinaceous odor.

ABSORBABLE SURGICAL SUTURE

Absorbable Surgical Suture, page 597—Change the first paragraph to read:

Absorbable Surgical Suture is a strand prepared from collagen derived from healthy mammals, purified, rendered sterile, and protected from contamination.

Change the *Description* to read:

Absorbable Surgical Suture is either a plain strand of collagen which has not been treated in any manner which will alter its normal rate of digestibility, known as *Type A, Plain or Untreated,* or a strand which has been tanned or otherwise treated so that it will resist digestion for longer but varying periods of time, and known, respectively, as *Type B, Mild Treatment; Type C, Medium Treatment;* and *Type D, Prolonged Treatment.* One form of treatment is frequently referred to as *Chromic.* The types mentioned are supplied as *Boilable* or as *Non-boilable* absorbable Surgical Suture.

NON-ABSORBABLE SURGICAL SUTURE

Non-absorbable Surgical Suture, page 599—Change the first sentence of the *Description* to read:

Non-absorbable Surgical Suture is a strand of material that effectively resists enzymatic digestion in living animal tissue.

INJECTIONS

Injections, page 725—Change the second paragraph to read:

Every care should be exercised in the preparation of Injections to prevent contamination. Good pharmaceutical practice requires also that each Injection, in its final container, be subjected individually to a physical inspection whenever the construction of the container permits.

Change the first paragraph in the section on *Containers for Injections,* page 726, to read:

The container and closure for the Injection shall not interact physically or chemically with the Injection in such manner as to alter the strength, quality or purity beyond the official requirement under the ordinary or customary conditions of handling, shipment, storage, sale or use. If the container is made of glass, it shall be clear and colorless or of light amber color and must permit inspection of the contents.

By authority of the U. S. P. Board of Trustees and of the Executive Committee of Revision.

ROBERT L. SWAIN, *Chairman*
U. S. P. Board of Trustees

LLOYD C. MILLER, *Director of*
Pharmacopeial Revision

Released June 13, 1952

United States Pharmacopoeial Convention

Second Annual Financial Statement Based upon the Report of the Auditor for the Year Ended December 31, 1951

STATEMENT OF INCOME AND EXPENSE (GENERAL FUND)

INCOME				
Sale of Pharmacopoeias:				
Collections	$142,377.79			
Less: Decrease in Accounts Receivable:				
December 31, 1950	$55,689.69			
December 31, 1951	6,948.80	48,740.89	$ 93,636.90	
Sale of Reference Standards, A. M. A. Articles, etc.:				
Collections		$ 15,984.09		
Add: Increase in Accounts Receivable:				
December 31, 1951	$ 1,510.85			
December 31, 1950	1,379.01	131.84		
		$ 16,115.93		
Less: Adjustment of prior year's collections	$ 64.17			
Refund of overpayments	42.08	106.25	16,009.68	
Interest on investments:				
Collections		$ 5,806.32		
Add: Increase in Accrued Interest Receivable:				
December 31, 1951	$ 1,499.97			
December 31, 1950	537.47	962.50		
		$ 6,768.82		
Less: Amortization of Bond Premium		37.18	6,731.64	
Use of Text by Others			1,085.00	
Miscellaneous			369.64	
TOTAL INCOME				$117,832.86
LESS—EXPENSE:				
Revision		$ 56,930.33		
Add: Decrease in Inventory:				
December 31, 1950	$18,944.91			
December 31, 1951	16,240.09	2,704.82	$ 59,635.15	
Administration			6,521.87	
Publication and Sales Expense		$ 3,396.40		
Add: Decrease in Inventory:				
December 31, 1950	$52,794.70			
December 31, 1951	26,849.92	25,944.78	29,341.18	
Convention			3,581.18	
Headquarters—New York		$ 4,659.20		
Add: New York Office Depreciation Expense:				
Building	$ 2,410.34			
Furniture and Equipment	2,614.08	5,024.42	9,683.62	
TOTAL EXPENSE				108,763.00
EXCESS OF INCOME OVER EXPENSE				$ 9,069.86

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

CASH ON DEPOSIT, December 31, 1950		$ 97,012.78
ADD—RECEIPTS:		
Sale of Pharmacopoeias	$142,377.79	
Sale of Reference Standards, A. M. A. Articles, etc.	15,984.09	
Interest on Investments	5,806.32	
Use of Text by Others	1,085.00	
Repayment from Building Fund	5,825.73	
Miscellaneous	369.64	171,448.57
		$268,461.35

(*Continued on page 460*)

LESS—DISBURSEMENTS:

	Revision	Administration	Publications	Convention	Headquarters	Total	
Printing and Binding	$.....	$.....	$3,396.40	$1,848.18	$.....	$ 5,244.58	
Salaries and Honoraria	20,124.00	2,384.00	.....	1,600.00	1,500.00	25,608.00	
Clerical	11,140.36	1,565.00	.....	.....	.....	12,705.36	
Meetings	6,671.95	1,278.89	.....	.....	.....	7,950.84	
Supplies	1,971.79	98.14	.....	.....	.....	2,069.93	
Postage and Telegrams	1,496.19	29.17	.....	133.00	.....	1,658.36	
Utilities	.....	.....	.....	.....	2,006.26	2,006.26	
Repairs and Maintenance	.....	.....	.....	.....	1,152.94	1,152.94	
General	15,526.04	1,166.67	.....	.....	.....	16,692.71	
Furnishings and Equipment	.....	.....	.....	.....	563.72	563.72	
Building Improvements	.....	.....	.....	.....	2,444.32	2,444.32	
Building Dedication Expense	.....	.....	.....	.....	200.00	200.00	
	$56,930.33	$6,521.87	$3,396.40	$3,581.18	$7,867.24		78,297.02
CASH ON DEPOSIT, December 31, 1951							$190,164.33

A MORE DETAILED AND SUPPLEMENTARY STATEMENT OF EXPENSES

REVISION MEETINGS		
Director's Conferences	$1,577.75	
Revision Committee	5,094.20	$ 6,671.95
REVISION GENERAL		
Retirement and Social Security	$6,388.21	
Reference Standards	6,523.86	
Exhibits	366.69	
Library Bindings	1,002.52	
Research Grant (Subcommittee on External Preparations)	750.00	
Miscellaneous	494.76	15,526.04
ADMINISTRATION GENERAL		
Auditor	$ 400.00	
Counsel	685.75	
Miscellaneous	80.92	1,166.67

CORRECTION

In the article entitled, "Studies on Species of *Asclepias*. VI. Toxicology, Pathology, and Pharmacology," by W. E. Hassan, Jr., and H. L. Reed [THIS JOURNAL, **41**, 298–300 (1952)], the caption which appears under Fig. 1 should read as follows: "Effects of prepared fluidextract of *Asclepias* upon uterus, blood pressure, and respiration of the cat. [After Costello, O. H., and Butler, C. L., THIS JOURNAL, **39**, 233(1950)]."

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